E.   ANALYSIS OF LIVER AND CO-EXPOSURE ISSUES FOR THE TCE TOXICOLOGICAL REVIEW

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CONTENTS—Appendix E: Analysis of Liver and Co-exposure Issues for the TCE Toxicological Review

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FOREWORD

The purpose of this Appendix is to provide scientific support and rationale for the hazard and dose-response sections regarding liver effects and those of co-exposure of the Toxicological Review of Trichloroethylene (TCE). It is not intended to be a comprehensive treatise on the chemical or toxicological nature of TCE. Please refer to the Toxicological Review of TCE for characterized of EPA's overall confidence in the quantitative and qualitative aspects of hazard and dose response for TCE-induce liver effects. Matters considered in this appendix include knowledge gaps, uncertainties, quality of data, and scientific controversies. This characterization is presented in an effort to make apparent the scientific issues regarding the data and MOA considerations for experimental animal data for liver effects in the TCE assessment.

For other general information about this assessment or other questions relating to IRIS, the reader is referred to U.S. EPA's IRIS Hotline at 301-345-2870.

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1. Basic physiology and function of the liver – a story of heterogeneity

The liver is a complex organ whose normal function and heterogeneity are key to understanding and putting into context perturbations by TCE, cancer biology, and variations in response observed and anticipated for susceptible life stages and background conditions.

1.1 Heterogeneity of Hepatocytes and Zonal Differences in Function and Ploidy

Malarkey et al 2005 state that (1) the liver transcriptome (i.e., genes expressed as measured by mRNA) is believed only second to the brain in its complexity and includes about 25-40% of the approximately 50,000 mammalian genes, (2) during disease states the transcriptome can double or triple and its increased complexity is due not only to differential gene expression (up- and down-regulation of genes) but also to the mRNA contributions from the heterogeneous cell populations in the liver and (3) when one considers that over a dozen cell types comprise the liver in varying proportions, particularly in disease states, knowledge about the cell types and cell-specific gene expression profiles help unravel the complex genomic and protenomic data sets. Gradients of gene and protein activity varying from the periportal region to the centrilobular region also exist for sinusoidal endothelial cells, Kuffper cells, hepatic stellate cells, and the matrix in the space of Disse. Malarkey et al (2005) also estimate that hepatocytes constitute 60%, sinusoidal endothelial cells 20%, Kupffer cells 15%, and stellate cells 5% of liver cells. Therefore in experimental paradigms where liver homogenates are used for the determination of "changes in liver" gene expression or other parameters the individual changes from cells residing in differing zones and by differing cell type is lost. Malarkey et al define the need to better characterize the histological cellular components of the tissues from which mRNA and protein is extracted and referred to "phenotypic anchoring" and cite acetaminophen as a "model hepatotoxicant under study to assess the strengths and weaknesses of genomics and proteinomics technologies" as well as "a good example for understanding and utilizing phenotypic anchoring to better understand genomics data." After acetaminophen exposure "there is an unexplained and striking inter and intralobular variability in acute hepatic necrosis with some regions having massive necrosis and adjacent areas within the same lobe or other lobes showing no injury at all." Malarkey et al. (2005) go on to cite similar lobular variability in response for "copper distribution, iron and phosphorous, chemical and spontaneous carcinogenesis, cirrhosis and regeneration" and suggest that although uncertain "factors such as portal streamlining of blood to the liver, redistribution of blood to core of the liver secondary to nerve stimulation, and exposures during fetal development and possibly lobular gradients are important." Hepatic interlobe differences exist for initiating agents in terms of DNA alkylation and cell replication. In the rat, DEN alkylation has been reported to occur preferentially in the left and right median lobes, while cell replication was higher in the right median and right anterior lobes (Richardson et al 1986). Richardson et al (1986) reported that exposure to DEN induced a 100% incidence of hepatocellular carcinoma in the left, caudate, left median and right

1   median lobes of the liver by 20 weeks versus only 30% in the right anterior and right posterior

2   hepatic lobes. There was a reported interlobe difference in adduct formation, cell proliferation,

3   liver lobe weight gain, number and size of GGT+ foci, and carbon 14 labeling from a single dose

4   of DEN.   Richardson et al. (1986) suggest that many growth-selection studies utilizing the liver

5   to evaluate the carcinogenic potential of a chemical often focus on only one or two of the hepatic

6   lobes, which is especially true for partial hepatectomy, and that for DEN and possibly other

7   chemicals this procedure removes the lobes most likely to get tumors. Thus, the "distribution of

8   toxic insult may not be correctly assessed with random sampling of the liver tissue for

9   microarray gene expression analysis" (Malarkey et al., 2005) and certainly any such

10   distributional differences are lost in studies of whole liver homogenates.

11   The liver is normally quiescent with few hepatocytes undergoing mitosis and, as

12   described below, normally occurring in the periportal areas of the liver. Mitosis is observed only

13   in approximately one in every 20,000 hepatocytes in adult liver (Columbano and Ledda-

14   Columbano, 2003). The studies of Schwartz-Arad et al. (1989), Zajicek et al. (1991), Zajicek

15   and Schwartz-Arad (1990), and Zajicek et al. (1989) have specifically examined the birth, death

16   and relationship to zone of hepatocytes as the "hepatic streaming theory." They report that

17   hepatocytes and littoral cells continuously steam from the portal tract toward the terminal hepatic

18   vein and that the hepatocyte differentiates as it goes with biological age closely related to cell

19   differentiation. In other words, the acinus may be represented by a tube with two orifices: for

20   cell inflow situated at the portal tract rim and other for cell outflow, at the terminal hepatic vein

21   with hepatocytes streaming through the tube in an orderly fashion. In normal liver, cell

22   proliferation is suggested as the only driving force of this flow with each mitosis associated with

23   displacement of the cells by one cell location and the greater the cell production, the faster the

24   flow and visa versa (Zajicek et al., 1991). Thus, the microscopical section of the liver "displays

25   an instantaneous image of a tissue in flux"(Schwartz-Arad et al., 1989). Schwartz-Arad et al

26   further suggest that "throughout its life the hepatocyte traverses three acinus zones; in each it is

27   engaged in different metabolic activity. When young it performs among other functions

28   gluconeogenesis, which is found in zone 1 hepatocytes (i.e. periportal), and when old it turns into

29   a zone 3 cell (i.e., pericentral), with a pronounced glycolitic make up. The three zones thus

30   represent differentiation stages of the hepatocyte, and since they differ by their distance from the

31   origin, e.g. zone 2 (i.e., midzonal) is more distant than zone 1, again, hepatocyte differentiation is

32   proportional to its distance."   Chen et al. (1995) report that "Hepatocytes are a heterogeneous

33   population that are composed of cells expressing different patterns of genes. For example,

34   gamma-glutamyl transpeptidase and genes related to gluconeogenesis are expressed preferential

35   in periportal hepatocytes, whereas enzymes related to glycolysis are more abundant in the

36   centrilobular area. Glutamine synthetase is expressed in a small number of hepatocytes

37   surrounding the central veins. Most cytochrome p450 enzymes are expressed or induced

38   preferentially in centrilobular hepatocytes relative to periportal hepatocytes." Along with

39   changes in metabolic function, Vielhauer et al. (2001) reported that there is evidence of zonal

40   differences in carcinogen DNA effects and also chemical specific differences for DNA repair

41   enzyme and that enhanced DNA repair is a general feature of many carcinogenic states including

42   the enzymes that repair alkylating agents but also oxidative repair. As part of this process of

1	differentiation and as livers age, the hepatocyte changes and increases its ploidy with polyploid
2	cells predominant in zone 2 of the acinus (Schwartz-Arad et al., 1989). The reported decrease in
3	DNA absorbance in zone 3 may be due to 1) a decline in chromatin affinity to the dye, 2) cell
4	death, and 3) DNA exit from intact cells and Zajicek and Schwartz-Arad (1990) suggest that the
5	fewer metabolic demands in Zone 3, under normal conditions, causes the cell to "deamplify" its
6	genes and for DNA excess to leak out cells adjacent to the terminal hepatic vein or to be
7	eliminated by apoptosis reflecting cell death. Thus, the three acinus zones represent
8	differentiation states of one and the same hepatocyte which increase ploidy as functional
9	demands change. Zajicek and Schwartz-Arad, (1990) also report that nuclear size is generally
10	proportional to DNA content and that as DNA accumulates, the nucleus enlarges. This has
11	import for histopathological descriptions of hepatocellular hypertrophy and attendant nuclear
12	changes after toxic insult as well.

13	The gene amplification associated with polyploidy is manifested by DNA accumulation
14	that involves the entire genome (Zajicek and Schwartz-Arad, 1990). Polyploidization is always
15	attended by the intensification of the transcription and translation and in rat liver the amino acid
16	label and activity of many enzymes increases proportionately to their ploidy. "Individual
17	chromosomes of a tetraploid genome of a hepatocyte reduplicate in the same sequence as in a
18	diploid one. In this case the properties of the chromosomes evidently remain unchanged and
19	polyploidy only means doubling the indexes of the diploid genome" (Brodsky and Uryvaeva
20	1977). Polyploidy will be manifest in the liver by either increases in the number of
21	chromosomes per nucleus in an individual cell, or by the appearance of two nuclei in a single
22	cell. Most cell polyploidization occurs in youth with mitotic polyploidization occurring
23	predominantly from 2 to 3 weeks post-natally and increases with age in mice (Brodsky and
24	Uryvaeva, 1977). Hepatocytes progress through a modified or polyploidizing cell cycle which
25	contains gaps and S-phases, but proceeds without cytokinesis. The result is the formation of the
26	first polyploidy cell which is binucleated with diploid nuclei and has increased cell ploidy but
27	not cell number. The subsequent proliferation of bi-nucleated hepatocytes occurs with a fusion
28	of mitotic nuclei during metaphase that gives rise to mononucleated cells with higher levels of
29	ploidy. Thus, during normal liver ontogenesis, a polyploidizing cell cycle without cytokinesis
30	alternates with a mitotic cycle of binucleated cells and results in progressive and irreversible
31	increases in either cell or nuclear ploidy (Brodsky and Uryvaeva 1977).

32	Polyploidization of the liver occurs during maturation in rodents and therefore
33	experimental paradigms that treat or examine rodent liver during that period should take into
34	consideration the normally changing baseline of polyploidy in the liver. The development of
35	polyploidy has been correlated in rodents to correspond with maturation. Brodsky and Uryvaeva
36	(1977) report it is cells with diploid nuclei that proliferate in young mice, but that among the
37	newly formed cells, the percentage of those with tetraploid nuclei is high. By 1 month most
38	mice (CBA/C57BL mice) already have a polyploid parenchyma, but binucleate cells with diploid
39	nuclei predominate. In adult mice the ploidy class with the highest percentage of hepatocytes
40	was the 4n X 2 class. The intensive proliferation of diploid hepatocytes occurs only in baby

1 mice during the first 2 weeks of life and then toward 1 month, the diploid cells cease to maintain
2 themselves and transform into polyploid cells. In aged animals the parenchyma retains only 0.02
3 percent of the diploid cells of the newborn animal. While the weight of the liver increases
4 almost 30 times within 2 years, the number of cells increases much less than the weight or mean
5 ploidy.   Hence the postnatal growth of the liver parenchyma is due to cell polyploidization
6 (Brodsky and Uryvaeva, 1977).   In male Wistar rats fetal hepatocytes (22 days gestation) were
7 reported to be 85.3% diploid (2n) and 7.4% polyploid (4n + 8n) cells with 7.3% of cells in S-
8 phase (S1 and S2). By one month of age (25- day old suckling rats) there were 92.9 % diploid
9 and 2.5% polyploid, at 2 months 47.5% diploid and 50.9% polyploid, at 6 months 29.1 % diploid
10 and 69.6% polyploid, and by 8 months 11.1 % diploid and 87.3% polyploidy (Sanz et al., 1996).
11 However, mouse and rat differ in their polyploidization. "In the mouse, which has a higher
12 degree of polyploidy than the rats, the scheme of polyploidization differs in that each cell class,
13 including mononucleate cells, forms from the preceding one without being supplemented by self-
14 maintenance. Each cell class is regarded as the cell clone and it is implied that the cells of each
15 class have the same mitotic history and originate from diploid initiator cells with similar
16 properties. In this model 1 reproduction would give a 2n X 2 cell, the second reproduction a 4n
17 cell, and third reproduction a 4n X 2 cell all coming from an originator diploid cell" (Brodsky
18 and Uryvaeva, 1977).

19   The cell polyploidy is most extensive in mouse liver, but also common for rat and
20 humans livers. The livers of young and aged mice differ considerably in the ploidy of the
21 parenchymal cells, but still perform fundamentally the same functions. In some mammals, such
22 as the mouse, rats, dog and human, the liver is formed of polyploid hepatocytes. In others, for
23 example, guinea pig and cats, the same functions are performed by diploid cells (Brodsky and
24 Uryvaeva, 1977). One obvious consequence of polyploidization is enlargement of the cells. The
25 volume of the nucleus and cytoplasm usually increases proportionately to the increased in the
26 number of chromosome sets with polyploidy reducing the surface/volume ratio. The labeling of
27 tritium doubles with the doubling of the number of chromosomes in the hepatocyte nucleus
28 (Brodsky and Uryvaeva, 1977). Kudryavtsev et al. (1993) have reported that the average levels
29 of cell and nuclear ploidy are relatively lower in humans than in rodent but the pattern of
30 hepatocyte polyploidization is similar and at maturity and especially during aging, the rate of
31 hepatocyte polyploidization increases with elderly individuals having binucleated and polyploid
32 hepatocytes constituting about one-half of liver parenchyma. Gramantieri et al. (1996) report
33 that in adult human liver a certain degree of polyploidization is physiological; the polyploidy
34 compartment (average 33% of the total hepatocytes) includes both mononucleated (28%) and
35 binucleated (72%) cells and the average percentage of binucleated cells in the total hepatocyte
36 population is 24% (Melchiorri et al., 1994). Historically, aging in human liver has been
37 characterized by fewer and larger hepatocytes, increased nuclear polyploidy and a higher index
38 of binucleate hepatocytes (Popper, 1986) but Schmucker (2005) notes that data concerning the
39 effect of aging on hepatocyte volume in rodent and humans are in conflict with some showing
40 increases volume to be unchanged and to increase by 25% by age 60 by others in humans. The
41 irreversibility of hepatocyte polyploidy has been used in efforts to identify the origin of tumor

1   progenitor cells (diploid vs. polyploidy) (see Section 3.1.8, below). The associations with
2   polyploidy and disease have been an active area of study in cancer MOA studies (see Sections
3   3.1.4. and 3.3.1, below).

4   Not only are polyploid cells most abundant in zone 2 of the liver acinus and increase in
5   number with age, but polyploid cells have been reported to be more abundant following a
6   number of toxic insults and exposure to chemical carcinogens. Wanson et al. (1980) reported
7   that one of the earliest lesions obtained in the liver after N-nitrosomorpholine (NNM) treatment
8   development of hypertrophic parenchymal cells presenting a high degree of ploidy. Gupta
9   (2000) reports hepatic polyploidy is often encountered in the presence of liver disease and that
10 for animals and people, polyploidy is observed during advancement of liver injury due to
11 cirrhosis or other chronic liver disease (often described as large-cell dysplasia referring to
12 nuclear and cytoplasmic enlargement, nuclear pleomorphisms and multinucleation and probably
13 representing increased prevalence of polyploidy cells) and in old animals with toxic liver injury
14 and impaired recovery.   Gorla et al. (2001) report that weaning and commencement of feeding,
15 compensatory liver hypertrophy following partial hepatectomy, toxin and drug-induced liver
16 disease, and administration of specific growth factors and hormones may induce hepatic
17 polyploidy. They go on to state that "although liver growth control has long been studied,
18 whether the replication potential of polyploidy hepatocytes is altered remains unresolved, in part,
19 owing to difficulties in distinguishing between cellular DNA synthesis and generation of
20 daughter cells." Following CCL4 intoxication, the liver ploidy rises and more cells become
21 binucleate (Zajicek et al., 1989).   Minamishima et al. (2002) report that in 8-12 week old female
22 mice before partial hepatectomy there were 78.6% 2C, 19.1% 4C, and 2.3% 8C cells but 7 days
23 after there were 42.0% 2C, 49.1% 4C, and 9.0% 8C. Zajicek et al. (1991) describe how
24 hepatocyte streaming is affected after the rapid hepatocyte DNA synthesis that occurs after the
25 mitogenic stimulus of a partial hepatectomy. These data are of relevance to findings of increased
26 DNA synthesis and liver weight gain following toxic insults and disease states. Zajicek et al.
27 (1991) suggest that following a mitogenic stimulus, not all DNA synthesizing cells do divide but
28 accumulate newly formed DNA and turn polyploid (i.e., during the first 3 days after partial
29 hepatectomy in rats 50% of synthesized DNA was accumulated) and that since the acinus
30 increased 15% and cell density declined 10%, overall cell mass increased 5%. However, cell
31 influx rose 1300%. "In order to accommodate all these cells, the 'acinus-tube' ought to swell 13-
32 fold, while in reality it increased only 5%" and that on day 3 "the liver remnant did not even
33 double in its size". Zajicek et al. conclude that apparently "cells were eliminated very rapidly,
34 and may have even been sloughed off, since the number of apoptotic bodies was very low" and
35 therefore "partial hepatectomy triggers two processes: an acute process lasting about a week
36 marked by massive and rapid cell turnover during which most newly formed hepatocytes are
37 eliminated, probably sloughed off into the sinusoids; and a second more protracted process
38 which served for liver mass restoration mainly by forming new acini." Thus, a mitogenic
39 stimulus may induce increased ploidy and increased cell number as a result of increased DNA
40 synthesis, and many of the rapidly expanding number of cells resulting from such stimulation are
41 purged and therefore do not participate in subsequent disease states of the liver.

1   Zajicek et al. (1989) note that the accumulation of DNA rather than proliferation of
2   hepatocytes "should be considered when evaluating the labeling index of hepatocytes labeled
3   with tritiated thymidine" as the labeling index, defined as the proportion of labeled cells, can
4   serve as a proliferation estimate only if it is assumed that a synthesizing cell will ultimately
5   divide. In tissues, such as the liver, "where cells also accumulate DNA, proliferation estimates
6   based on this index may fail" (Zajicek et al., 1989). The tendency to accumulate DNA is also
7   accompanied by a decreasing probability of a cell to proliferate, since young hepatocytes
8   generally divide after synthesizing DNA while older cells prefer instead to accumulate DNA.
9   However, polyploidy *per se* does not preclude cells from dividing (Zajicek et al., 1989). The
10   ploidy level achieved by the cell, no matter how high, does not in itself prevent it from going
11   through the next mitotic cycle and the reproduction of hepatocytes in the ploidy classes of 8n and
12   8n X2 is common phenomenon (Brodsky and Uryvaeva, 1977). However, along with a reduced
13   capacity to proliferate, Sigal et al. (1999) report that the onset of polyploidy increases the
14   probability of cell death. The proliferative potentials of hepatocytes not only depend on their
15   ploidy, but also on the age of the animals with liver restoration occurring more slowly in aged
16   animals after partial hepatectomy (Brodsky and Uryvaeva, 1977).   Species differences in the
17   ability of hepatocytes to proliferate and respond to a mitogenic stimulus have also been
18   documented (see Section 3.4.2, below). The importance of the issues of cellular proliferation vs.
19   DNA accumulation and the differences in ability to respond to a mitogenic stimulus becomes
20   apparent as identification of the cellular targets of toxicity (i.e., diploid vs. polyploidy) and the
21   role of proliferation in proposed modes of action (MOA) are brought forth. Polyploidization , as
22   discussed above, has been associated with a number of types of toxic injury, disease states, and
23   carcinogenesis by a variety of agents.

24   1.2. Effects of Environment and Age: Variability of Response

25   The extent of polyploidization of the liver not only changes with age, but structural and
26   functional changes, as well as environmental factors (e.g., polypharmacy), affect the
27   vulnerability of the liver to toxic insult. In a recent review by Schmucker (2005), several of
28   these factors are discussed. Schmucker reports that approximately 13% of the population of the
29   United States is over the age of 65 years, that the number will increase substantially over the next
30   50 years, and that increased age is associated with an overall decline in health and vitality
31   contributing to the consumption of nearly 40% of all drugs by the elderly. Schmucker estimates
32   that 65% of this population is medicated and many are on polypharmacy regimes with a major
33   consequence of a marked increase in the incidence of adverse drug reactions (ADRs) (i.e., males
34   and females exhibit 3- and 4-fold increases in ADRs, respectively, when 20- and 60-year old
35   groups are compared). The percentage of deaths attributed to liver diseases dramatically
36   increases in humans beyond the age of 45 years with data from California demonstrating a 4-fold
37   increase in liver disease-related mortality in both men and women between the ages of 45 and 85
38   years (Seigel and Kasmin, 1997). Furthermore, Schmucker cites statistics from the United Stated
39   Department of Health and Human Services to illustrate a loss in potential lifespan prior to 75
40   years of age due to liver disease (i.e., liver disease reduced lifespan to a greater extent than
41   colorectal and prostatic cancers, to a similar extent as chronic obstructive pulmonary disease, and

1   nearly as much as HIV). Thus, the elderly are predisposed to liver disease.

2   As stated above, the presence of high polyploidy cell in normal adults, nuclear
3   polyploidization with age, and increase in the mean nuclear volume have been reported in
4   people. Wantanabe et al. (1978) reported the results from a cytophotometrical analysis of 35
5   cases of sudden death including 22 persons over 60 years of age, that revealed that although the
6   nuclear size of most hepatocytes in a senile liver remains unchanged, there was an increase in
7   cells with larger nuclei. Variations in both cellular area and nucleocytoplasmic ratio were also
8   analyzed in the study, but the binuclearity of hepatocytes was not considered. No cases with a
9   clinical history of liver disease were included. Common changes in senile liver were reported to
10   include atrophy, fatty metamorphosis of hepatocytes, and occasional collapse of cellular cords in
11   the centrilobular area, slight cellular infiltration and proliferation of Kupffer cells in sinusoids,
12   and elongation of Glisson's triads with slight to moderate fibrosis in association with round cell
13   infiltration.   Furthermore, cells with giant nuclei, with each containing two or more prominent
14   nucleoli, and binuclear cell. There was a decrease in diploid populations with age and an
15   increase in tetraploid population and a tendency of polyploidy cells with higher values than
16   hexaploids with age. Cells with greater nuclear size and cellular sizes were observed in livers
17   with greater degrees of atrophy.

18   Schmucker notes that one of the most documented age-related changes in the liver is a
19   decline in organ volume but also cites a decrease in functional hepatocytes and that other studies
20   have suggested that the size or volume of the liver lobule increases as a function of increasing
21   age. Data is cited for rats suggesting sinusoidal perfusion rate in the rat liver remains stable
22   throughout the lifespan (Vollmar et al., 2002) but evidence in humans shows age-related shifts in
23   the hepatic microcirculation attributable to changes in the sinusoidal endothelium (McLean et al.,
24   2003) (i.e., a 60% thickening of the endothelial cell lining and an 80% decline in the number of
25   endothelial cell fenestrations, or pores, with increasing age in humans) that are similar in baboon
26   liver (Cogger et al., 2003). Such changes could impair sinusoidal blood flow and hepatic
27   perfusion, and the uptake of macromolecules such as lipoproteins from the blood. Schmucker
28   reports that there is a consensus that hepatic volume and blood flow decline with increasing age
29   in humans but that the effects of aging on hepatocyte structure are less clear. In rats the volume
30   of individual hepatocytes was reported to increase by 60% during development and maturation,
31   but subsequently decline during senescence yielding hepatocytes of equivalent volumes in
32   senescent and very young animals (Schmucker, 2005). The smooth surfaced endoplasmic
33   reticulum (SER), which is the site of a variety of enzymes involved in steroid, xenobiotic, lipid
34   and carbohydrate metabolism, also demonstrated a marked age-related decline rat hepatocytes
35   (Schumucker et al., 1977: Schmucker et al., 1978). Schmucker also notes that several studies
36   have reported that the older rodents have less effective protection against oxidative injury in
37   comparison to the young animals, age-related decline in DNA base excision repair, and increases
38   in the level of oxidatively damaged DNA in the livers of senescent animals in comparison to
39   young animals.   Age-related increases in the expression an activity of stress-induced
40   transcription factors (i.e., increased NF-κB binding activity but not expression) were also noted,

1 but that the importance of changes in gene expression to the role of oxidative stress in the aging
2 process remains unsolved. An age-related decline in the proliferative response of rat hepatocytes
3 to growth factors following partial hepatectomy was noted, but despite a slower rate of hepatic
4 regeneration, older livers eventually achieved their original volume with the mechanism
5 responsible for the age-related decline in the post-hepatectomy hepatocyte proliferative response
6 unidentified. As with other tissues, telomere length has been identified as a critical factor in
7 cellular aging with the sequential shortening of telomeres to be a normal process that occurs
8 during cell replication (see Sections 3.1.1 and 3.1.7, below). An association in telomere length
9 and strain susceptibility for carcinogenesis in mice has been raised. Herrera et al., (1999)
10 examined susceptibility to disease with telomere shortening in mice. However, this study only
11 cites shorter telomeres for C57BL6 mice in comparison to mixed C57BL6/129sv mice. The
12 actual data are not in this paper and no other strains are cited. Of the differing cell types
13 examined, Takubo and Kaminishi (2001) report that hepatocytes exhibited the next fastest rate of
14 telomere shortening despite being relatively long-lived cells raising the question of whether or
15 not there are correlations between age, hepatocyte telomere length and the incidence of liver
16 disease (Schmucker 2005). Aikata et al. (2000) and Takubo et al. (2000) report that the mean
17 telomere length in healthy livers is approximately 10 kilobase pairs at 80 years of age and these
18 hepatocytes retain their proliferative capacity but that in diseased livers of elderly subjects was
19 approximately 5 kb pairs. Thus, short telomere length may compromise hepatic regeneration and
20 contribute to a poor prognosis in liver disease or as a donor liver (Schmucker, 2005).

21   Schmucker (2005) reports that interindividual variability in Phase I drug metabolism was
22 so large in human liver microsomes, particularly among older subjects, that the determination of
23 any statistically significant age or gender-related differences were precluded. In fact Schmucker
24 (2001) notes that "the most remarkable characteristic of liver function in the elderly is the
25 increase in interindividual variability, a feature that may obscure age-related differences".
26 Schumer notes that The National Institute on Aging estimates that only 15% of individuals aged
27 over 65 years exhibit no disease or disability with this percentage diminishing to 11% and 5% for
28 men and women respectively over 80 years. Thus, the large variability in response and the
29 presence of age-related increases in pharmacological exposures and disease processes are
30 important considerations in predicting potential risk from environmental exposures.
31
32 ## 2.   Characterization of Hazard From TCE Studies
33
34   The 2001 Draft assessment of the health risk assessment of TCE (U.S. EPA, 2001)
35 extensively cited the review article by Bull (2000) to describe the liver toxicity associated with
36 TCE exposure in rodent models. Most of the attention has been paid to the study of TCE
37 metabolites, rather than the parent compound, and the review of the TCE studies by Bull (2000)
38 was cursory. In addition, gavage exposure to TCE has been associated with a significant
39 occurrence of gavage-related accidental deaths and vehicle effects, and TCE exposure through
40 drinking water has been reported to decrease palatability and drinking water consumption, and to
41 have significant loss of TCE through volatilization, thus, further limiting the TCE database. In

1   its review of the draft assessment, EPA's Science Advisory regarding this topic suggested that in
2   its revision, the studies of TCE should be more fully described and characterized, especially
3   those studies considered to be key for the hazard assessment of TCE. Although the database for
4   studies of the parent compound is somewhat limited, a careful review of the rodent studies
5   involving TCE can bring to bring to light the consistency of observations across these studies,
6   and help inform many of the questions regarding potential MOAs of TCE toxicity in the liver.
7   Such information can inform current MOA hypothesis (e.g., such as PPARα activation) as well.
8   Accordingly the primary acute, subchronic and chronic studies of TCE will be described and
9   examined in detail below and with comments on consistency, major conclusions and the
10   limitations and uncertainties that their design and conduct. Since all chronic studies were
11   conducted primarily with the goal of ascertaining carcinogenicity, their descriptions focus on that
12   endpoint, however, any noncancer endpoints described by the studies are described as well. For
13   details regarding evidence of hepatotoxicity in humans and associations with increased risk of
14   hepatocellular carcinoma, please refer to Section XXX of the 2008 revised assessment. Given
15   that some of the earlier studies with TCE were contaminated with epichlorhydrin, only the ones
16   without such contamination are examined below.
17
18   ## 2.1 Acute Toxicity Studies.
19
20   A number of acute studies have been undertaken to describe the early changes in the liver
21   after TCE administration with the majority using the oral gavage route of administration. Some
22   have been detailed examinations while others have reported primarily liver weight changes as a
23   marker of TCE-response. The matching and recording of age but especially initial and final
24   body weight for control and treatment groups is of particular importance for studies using liver
25   weight gain as a measure of TCE-response as difference in these parameter affect TCE-induced
26   liver weight gain. Most data are for exposures of at least 10 days.
27
28   **2.1.1. Soni et al. 1998**
29
30   Soni et al. (1998) administered TCE in corn oil to male Sprague-Dawley rats (200-250 g,
31   8-10 weeks old) intraperitoneally at exposure levels of 250, 500, 1250, and 2500 mg/kg. Groups
32   (4-6 animals per group) were sacrificed at 0, 6, 12, 24, 36, 48, 72, and 96 hours after
33   administration of TCE or corn oil. Using this paradigm only 50% of rats survived the 2400
34   mg/kg ip TCE administration with all deaths occurring between days 1 and 3 after TCE
35   administration. Tritiated thymidine was also administered i.p. to rats 2 hours prior to euthanasia.
36   Light microscopic sections of the central lobe in 3-4 sections examined for each animal. The
37   grading scheme reported by the authors was: 0 , no necrosis; +1 minimal, defined as only
38   occasional necrotic cells in any lobule; +2, mild, defined as less than one-third of the lobular
39   structure affected; +3, moderate, defined as between one-third and two-thirds of the lobular
40   structure affected; +4 severe, defined as greater than two-thirds of the lobular structure affected.
41   At the 2500 mg/kg dose histopathology data were obtained for the surviving rats (50%).
42   Lethality studies were done separately in groups of 10 rats. The survival in the groups of rats

1   administered TCE and sacrificed from 0 to 96 hours was given as 30% mortality at 48 hours and
2   50% mortality by 72 hours.
3
4   The authors report that controls and 0-hour groups did not show sign of tissue injury or
5   abnormality. The authors only report a single number with one significant figure for each group
6   of animals with no means or standard deviations provided. In terms of the extent of necrosis
7   there is no difference between the 250 and 500 mg/kg/treated dose groups though 96 hours with
8   a single +1 given as the maximal amount of hepatocellular necrosis (minimal as defined by
9   occasional necrotic cells in any lobule). At the 1250 mg/kg dose the maximal score was
10   achieved 24 hours after TCE administration and was reported as simply +2 (mild, defined as less
11   than one-third of lobular structure affected).   The level of necrosis was reported to diminish to a
12   score of 0 72 hours after 250 mg/kg TCE with no decrease at 500 mg/kg. At 1250 mg/kg the
13   extent of necrosis was reported to diminish from +2 to +1 by 72 hours after administration.   At
14   the 2500 mg/kg dose (LD50 for this route) by 48 hours the surviving rats were reported to have a
15   score of +4 (severe as defined by greater than two thirds of the lobular structure affected).   The
16   authors report that "The necrosed cells were concentrated mostly in the midzonal areas and the
17   cells around central vein area were unaffected. Extensive necrosis was observed between 24 and
18   48 hours for both 1250 and 2500 mg/kg groups. Injury was maximal in the group receiving 2500
19   mg/kg between 36 and 48 hours as evidenced by severe midzonal necrosis, vacuolization, and
20   congestion. Infiltration of polymorphonuclear cell was evident at this time as a mechanism for
21   cleaning dead cells and tissue debris from the lobules. At the highest dose, the injury also started
22   to spread toward the centrilobular areas. At highest dose 30 and 50% lethality was observed at 48
23   and 72 h, respectively. After 48 h, the number of necrotic cells decreased and the number of
24   mitotic cells increased. The groups receiving 500 and 1250 mg/kg TCE showed relatively higher
25   mitotic activity as evidenced by cells in metaphase compared to other groups." The authors do
26   not give a quantitative estimate or indication as to the magnitude of the number of cells going
27   through mitosis. Although there was variability in the number of animals dying at 1250 mg/kg
28   TCE exposure though this route of exposure, no indication of variability in response within these
29   treatment groups is given by the author in regard to extent of histopathological changes. The
30   authors do not comment on the manner of death using this paradigm or of the effects of i.p.
31   administration regarding potential peritonitis and inflammation.
32
33   TCE hepatotoxicity was "assessed by measuring plasma" sorbitol dehydrogenase (SDH)
34   and alanine aminotransferase (ALT) after TCE administration with vehicle treated control groups
35   reported to induce no increases in these enzymes. Plasma SDH levels were reported to increase
36   in a linear fashion after 250, 500 and 1250 mg TCE/kg ip administration by 6 hours (i.e., ~ 3-
37   fold, 10.5-fold, 22-fold, and 24.5-fold in comparison to controls from 250, 500, 1250, and 2500
38   mg/kg TCE, respectively) with little difference between the 1250 mg/kg and 250 mg/kg dose.
39   By 12 hours the 250, 500, and 1250 levels has diminished to levels similar to that of the 250
40   mg/kg dose at 6 hours. The 2500 mg/kg levels was somewhat diminished from its 6 hour level.
41   By 24 hours after TCE administration by the i.p. route of administration all doses were similar to
42   that of 250 mg/kg TCE 6 hour level. This pattern was reported to be similar for5 36, 48, 72, and
43   96 hour time points as well. The results presented were the means and SE for four rats per

1   group. The authors did not indicate which rats were selected for these results from the 4-6 that
2   were exposed in each group. Thus, only SDH levels showed dose dependence in results at the 6
3   hour time point and such increases did not parallel the patterns reported for hepatocellular
4   necrosis from histopathological examination of liver tissues.
5
6   For ALT, the pattern of plasma concentrations after i.p. TCE administration differed both
7   from that of SDH but also from liver histopathology.   Plasma ALT levels were reported to
8   increase in a nonlinear fashion and to a much smaller extent that SDH (i.e., ~ 2.7-fold, 1.9-fold,
9   2.1-fold, and 4.0-fold of controls from 250, 500, 1250, and 2500 mg/kg TCE, respectively).   The
10   patterns for 12, 24, 36, 48, 72, and 96 hours were similar to that of the 6 hour exposure and did
11   not show a dose-response. The authors injected carbon tetrachloride (2.5.ml/kg) into a separate
12   group of rats and then incubated the resulting plasma with unbuffered TCA (0, 200, 600, or 600
13   nmol and go decreases in enzyme activity in vitro at the two higher concentrations.   It is not
14   clear whether in vitro unbuffered TCE concentrations of this magnitude, which could precipitate
15   proteins and render the enzymes inactive, are relevant to the patterns observed in the in vivo
16   data. The extent of extinguishing of SDH and ALT activity at the two highest TCA levels in
17   vitro were the same, suggestive of the generalized in vitro pH effect. However, the enzyme
18   activity levels after TCE exposure had different patterns, and thus suggesting that *in vitro* TCA
19   results are not representative of the *in vivo* TCE results. Neither ALT nor SDH levels
20   corresponded to time course or dose-response reported for the histopathology of the liver
21   presented in this study.
22
23   Tritiated thymidine results from isolated nuclei in the liver did not show a pattern
24   consistent with either the histopathology or enzyme results. These results were for whole liver
25   homogenates and not separated by nuclear size or cell origin. Tritiated thymidine incorporation
26   was assumed by the authors to represent liver regeneration. There was no difference between
27   treated and control animals at 6 hours after i.p. TCE exposure and only a decrease (~ 50%
28   decrease) in thymidine incorporation after 12 hours of the 2500 mg/kg TCE exposure level. By
29   24 hours, there as 5.6-fold and 2.8- fold tritiated thymidine incorporation at the 500 and 1250
30   mg/kg TCE levels with the 250 and 2500 mg/kg levels similar to controls.   For 36, 48, and 72
31   hours after i.p. TCE exposure there continued to be no dose-response and no consistent pattern
32   with enzyme or histopathological lesion patterns.   The authors presented "area under the curve"
33   data for tritiated thymidine incorporation for 0 to 95 hours which did not include control values.
34   There was a slight elevation at 500 mg/kg TCE and slight decrease at 2500 mg/kg from the 250
35   mg/kg TCE levels. Again, these data did not fit either histopathology or enzyme patterns and
36   also can include the contribution of nonparenchymal cell nuclei as well as changes in ploidy.
37
38   The use of an i.p route of administration is difficult to compare to oral and inhalation
39   routes of exposure given that peritonitis and direct contact with TCE and corn oil with liver
40   surfaces may alter results. Whereas Soni et al. (1998) report the LD50 to be 2500 mg/kg TCE
41   via i.p administration, both Elcombe et al. (1985) and Melnick et al. (1987) do not report
42   lethality from TCE administered for 10 days at 1500 mg/kg in corn oil, or up to 4800 mg/kg/day
43   for 10-days in encapsulated feed. Also TCE administered via gavage or oral administration

1   through feed will enter the liver through the circulation with periportal areas of the liver the first
2   areas exposed with the entire liver exposed in a fashion dependent on blood concentrations
3   levels. However, with i.p administration, the absorption and distribution pattern of TCE will
4   differ. The lack of concordance with measures of liver toxicity from this study and the lack
5   concordance of patterns and dose-response relationships of toxicity reported from other more
6   environmentally and physiologically relevant routes of exposure make the relevance of these
7   results questionable.
8
9   **2.1.2. Soni et al. 1999.**
10
11   A similar paradigm and the same results were reported for Soni et al (1999), in which
12   hepatocellular necrosis, tritiated thymidine incorporation, and in vitro inhibition of SDH and
13   ALT data were presented along with dose-response studies with ally alcohol and a mixture of
14   TCE, Thioacetamine, allyl alcohol, and chloroform. The same issues with interpretation present
15   for Soni et al. (1998) also apply to this study as well.
16
17   **2.1.3. Okino et al. 1991**
18
19   This study treated adult Wistar male rats (8 weeks of age) with TCE after being on a
20   liquid diet for 3 weeks and either untreated or pretreated with phenobarbital or ethanol. TCE
21   exposure was at 8000 ppm for 2 hours, 2000 or 8000 ppm for 2 hours, and 500 or 2000 ppm for
22   8 hours. Each group contained 5 rats. Livers from rats that were not pre-treated with either
23   ethanol or phenobarbital were reported to show only a few necrotic hepatocytes around the
24   central vein at 6 and 22 hours after 2 hours of 8000 ppm TCE exposure. At increased lengths
25   and/or concentrations of TCE exposure, the frequencies of necrotic hepatocytes in the
26   centrilobular area were reported to be increased but the number of necrotic hepatocytes was still
27   relatively low (out of ~150 hepatocytes the percentages of necrotic pericentral hepatocytes were
28   0.2 ± 0.4, 0.3 ± 0.4, 2.7 ± 1.0, 0.2 ± 0.4, and 3.5 ± 0.4 % for control, 2000 ppm TCE for 2 hours,
29   8000 ppm TCE for 2 hours, 500 ppm TCE for 8 hours, and 2000 ppm TCE for 8 hours,
30   respectively). "Ballooned" hepatocytes were reported to be zero for controls and all TCE
31   treatments with the exception of 0.3 ± 0.6 % ballooned midzonal hepatocytes after 8000 ppm
32   TCE for 2 hours exposure. Microsomal protein (mg/g/liver) was increased with TCE exposure
33   concentration and duration, but not reported to be statistically significant (mg/g/liver microsomal
34   protein was 21.2 ± 4.3, 22.0 ± 1.5, 25.9 ± 1.3, 23.3 ± 0.8, and 24.1 ± 1.0 for control, 2000 ppm
35   TCE for 2 hours, 8000 ppm TCE for 2 hours, 500 ppm TCE for 8 hours, and 2000 ppm TCE for
36   8 hours, respectively). The metabolic rate of TCE was reported to be increased after exposures
37   over 2000 ppm TCE (metabolic rate of TCE in nmol/g/liver/min was 29.5 ± 5.7, 51.3 ± 6.0, 63.1
38   ± 16.0, 37.3 ± 3.3, and 69.5 ± 4.3 for control, 2000 ppm TCE for 2 hours, 8000 ppm TCE for 2
39   hours, 500 ppm TCE for 8 hours, and 2000 ppm TCE for 8 hours, respectively). However, the
40   cytochrome P450 content of the liver was not reported to increase with TCE exposure
41   concentration or duration. The liver/body weight ratios were reported to increase with all TCE
42   exposures except 500 ppm for 8 hours (the liver/ body weight ratio was 3.18 ± 0.15, 3.35 ± 0.10,
43   3.39 ± 0.20, 3.15 ± 0.10, and 3.57 ± 0.14 for control, 2000 ppm TCE for 2 hours, 8000 ppm TCE

1 for 2 hours, 500 ppm TCE for 8 hours, and 2000 ppm TCE for 8 hours, respectively). These
2 values represent 1.05-fold, 0.99-fold, 1.06-fold, and 1.12-fold of control in the 2000 ppm TCE
3 for 2 hours, 8000 ppm TCE for 2 hours, 500 ppm TCE for 8 hours, and 2000 ppm TCE for 8
4 hours treatment groups, respectively, with a statistically significant difference observed after 8
5 hours of 2000 ppm TCE exposure. Initial body weights and those 22 hours after cessation of
6 exposure were not reported, which may have affected liver weight gain. However, these data
7 suggest that TCE-related increases in metabolism and liver weight occurred as early as 22 hours
8 after exposures of this magnitude from 2 to 8 hours of TCE with little concurrent hepatic
9 necrosis.
10
11   Ethanol and phenobarbital pretreatment were reported to enhance TCE toxicity. In
12 ethanol-treated rats a few necrotic hepatocytes were reported to be around the central vein along
13 with hepatocellular swelling without pyknotic nuclei at 6 hours after TCE exposure with no
14 pathological findings in the midzonal or periportal areas. At 22 hours centrilobular hepatocytes
15 were reported to have a few necrotic hepatocytes and cell infiltrations around the central vein but
16 midzonal areas were reported to have ballooned hepatocytes with pyknotic nuclei frequently
17 accompanied by cell infiltrations. In phenobarbital treated rats 6 hours after TCE exposure,
18 centrilobular hepatocytes showed prenecrotic changes with no pathological changes reported to
19 be observed in the periportal areas. By 22 hours zonal necrosis was reported in centrilobular
20 areas or in the transition zone between centrilobular and periportal areas. Treatment with
21 phenobarbital or ethanol induced hepatocellular necrosis primarily in centrilobular areas with
22 phenobarbital having a greater effect (89.1 % ± 8.5% centrilobular necrosis) at the higher dose
23 and shorter exposure duration (8000 ppm x 2 hours) and ethanol having a greater effect (16.8 ±
24 5.3% centrilobular necrosis) at the lower concentration and longer duration of exposure (2000
25 ppm x 8 hours).
26
27 **2.1.4. Nunes et al. 2001**
28
29   This study was focused on the effects of TCE and lead co-exposure but treated male 75-
30 day old Sprague Dawley rats with 2000 mg/kg TCE for 7 days via corn-oil gavage (n= 10). The
31 rats ranged in weight from 293 to 330 g (~12%) at the beginning of treatment and were
32 pretreated with corn oil for 9 days prior to TCE exposure. TCE was reported to be 99.9% pure.
33 Although the methods section states that rats were exposed to TCE for 7 days, Table 1 of the
34 study reports that TCE exposure was for 9 days. The beginning body weights were not reported
35 specifically for control and treatment groups, but the body weights at the end of exposure were
36 reported to be 342 ± 18 g for control rats and 323 ± 3 g for TCE exposed rats, and that difference
37 (~6%) to be statistically significant. Because beginning body weights were not reported, it is
38 difficult to distinguish whether differences in body weight after TCE treatment were treatment
39 related or reflected differences in initial body weights. The liver weights were reported to be
40 12.7 ± 1.0 g in control rats and 14.0 ± 0.8 g for TCE treated rats with the % liver/body weight
41 ratios of 3.7% and 4.3 %, respectively.   The increase in % liver/body weight ratio represents
42 1.16-fold of control and was reported to be statistically significant. However, difference in
43 initial body weight could have affected the magnitude of difference in liver weight between

1 control and treatment groups. The authors report no gross pathological changes in rats gavaged
2 with corn oil or with corn oil plus TCE but observed that one animal in each group had slightly
3 discolored brown kidneys.   Histological examinations of "selected tissues" were reported to
4 show an increased incidence of chronic inflammation in the arterial wall of lungs from TCE-
5 dosed animals. There were no descriptions of liver histology given in this report for TCE-
6 exposed animals or corn-oil controls.
7
8 **2.1.5. Tao et al. 2000**
9
10   The focus of this study was to assess the affects of methionine on methylation and
11 expression of c-Jun and C-Myc in mouse liver after 5 days of exposure to TCE (1000 mg/kg in
12 corn oil) and its metabolites. Female 8-week old B6C3F1 mice (n= 4-6) were administered TCE
13 ("molecular biology or HPLC grade") for 5 days with and without methionine (300 mg/kg i.p).
14 Data regarding % liver/body weight was presented as a figure. Of note is the decrease in
15 liver/body weight ratio by methionine treatment alone (~ 4.6 % liver/body weight for control and
16 ~ 4.0 % liver/body weight for control mice with methionine or ~ 13% difference between these
17 groups). Neither initial body weights or body weights after exposure were reported by the
18 authors so that the reported effects of treatment could have reflected differences in initial body
19 weights of the mice. TCE exposure was reported to increase the % liver/body weight ratio to ~
20 5.8 % without methionine and to increase % liver/body weight ratio to ~ 5.7 % with methionine
21 treatment. These values represent 1.26-fold of control levels from TCE exposure without
22 methionine and 1.43-fold of control from TCE exposure with methionine. The number of
23 animals examined was reported to be 4-6 per group. The authors reported the differences
24 between TCE treated animals and their respective controls to be statistically significant but did
25 not examine the differences between controls with and without methionine. There were no
26 descriptions of liver histology given in this report for TCE-exposed animals or corn-oil controls.
27
28 **2.1.6. Tucker et al. 1982**
29
30   This study describes acute LD50, and 5- and 14-days studies of TCE in a 10% emulphor
31 solution administered by gavage. Screening level subchronic drinking water experiments with
32 TCE dissolved in 1% emulphor in mice were also conducted but with little detail reported. The
33 authors did describe the strains used (CD-1 and ICR outbred albino) and that they are "weanling
34 mice," but the ages of the mice and their weights were not given. The TCE was described as
35 containing 0.004% diisopropylamine as the preservative and that the stabilizer had not been
36 found carcinogenic or overtly toxic. The authors report that "the highest concentration a mouse
37 would receive during these studies is 0.03 mg/kg/day". The main results are basically an LD50
38 study and a short term study with limited reporting for 4 and 6-month studies of TCE.
39 Importantly, the authors documented the loss of TCE from drinking water solutions (less than
40 20% of the TCE was lost during the 3 or 4 days in the water bottles at 1.0, 2.5 and 5.0 mg/ml
41 concentrations, but in the case of 0.1 mg/ml, up to 45% was lost over a 4-day period). The
42 authors also report that high doses of TCE in drinking water reduced palatability to such an
43 extent that water consumption by the mice was significantly decreased.

1   The LD50 with 95% confidence were reported to be 2443 mg/kg (1839 to 3779) for
2   female mice and 2402 mg/kg (2065 to 2771) for male mice. However, the number of mice used
3   in each dosing group was not given by the authors. The deaths occurred within 24 hours of TCE
4   administration and no animals recovering from the initial anesthetic effect of TCE died during
5   the 14-day observation period. The authors reported that the only gross pathology observed was
6   hyperermia of the stomach of mice dying form lethal doses of TCE, and that mice killed at 14
7   days showed not gross pathology. In a separate experiment, male CD-1 mice were exposed to
8   TCE by daily gavage for 14 days at 240 and 24 mg/kg. These two doses did not cause treatment
9   related deaths and body weight and "most" organ weights were reported by the authors to not be
10   significantly affected but the data was not shown. The only effect noted was an increased liver
11   weight which appeared to be dose dependent but was reported to be significant only at the higher
12   dose. The only significant difference found in hematology was s 5% lower hematocrit in the
13   higher dose group. The number of animals tested in this experiment was not give by the authors.
14   Male CD-1 mice (n=11) were given TCE via gavage for 5 days (0.73 g/kg TCE twice on day 0,
15   1.46 g/kg twice on day 1, 2.91 g/kg twice on day 3 and 1.46 g/kg TCE on days 4 and 5) with
16   only 4 of 11 mice treated with TCE surviving.
17
18   In a subchronic study, male and female CD-1 mice received TCE in drinking water at
19   concentrations of 0, 0.1, 1.0, 2.5 and 5 mg/ml in 1% emulphor, and a naïve group received
20   deionized water. There were 140 animals of each sex in the naïve group and in each treatment
21   group, except for 260 mice in the vehicle groups. Thirty mice of each sex and treatment were
22   selected for recording body weights for 6 months. The method of "selection" was not given by
23   the authors. These mice were weighed twice weekly and fluid consumption was measured by
24   weighing the six corresponding water bottles. The authors reported that male mice at the two
25   highest doses of TCE consumed 41 and 66 ml/kg/day less fluid over the 6 months of the study
26   than mice consuming vehicle only and that this same decreased consumption was also seen in the
27   high dose (5 mg/ml) females. They report that weight gain was not affected except at the high
28   dose (5mg/ml) and even though the weight gain for both sexes was lower than the vehicle
29   control group, it was not statistically significant but this data was not shown. The authors report
30   that gross pathological examinations performed on mice killed at 4 and 6 months were
31   unremarkable and that a number of mice from all the dosing regimens had liver abnormalities,
32   such as pale, spotty, or granular livers. They report that 2 of 58 males at 4 months, and 11 of 59
33   mice at 6 months had granular livers and obvious fatty infiltration, and that mice of both sexes
34   were affected. Animals in the naïve and vehicle groups were reported to infrequently have pale
35   or spotty livers, but exhibit no other observable abnormalities. No quantitation or more detailed
36   descriptions of the incidence of or severity of effects were given in this report.
37
38   The average body weight of male mice receiving the highest dose of TCE was reported to
39   be 10% lower at 4 months and 11% lower at 6 months with body weights of female mice at the
40   highest dose also significantly lower. Enlarged livers (as percentage of body weight) were
41   observed after both durations of exposure in males at the three highest doses, and in females at
42   the highest dose. In the 4 month study, brain weights of treated females were significantly
43   increased when compared to vehicle control. However, the authors state that "this increase is

1   apparently because the values for the vehicle group were low, because the naïve group was also
2   significantly increased when compared to vehicle control." "A significant increase in kidney
3   weight occurred at the highest dose in males at 6 months and in females, after both 4 and 6
4   months of TCE exposure. Urinalysis indicated elevated protein and ketone levels in high-dose
5   females and the two highest dose males after 6 months of exposure (data not shown)." The
6   authors describe differences in hematology to include "a decreased erythrocyte count in the high
7   dose males at 4 and 6 months (13% and 16%, respectively); decreased leukocyte counts,
8   particularly in the females at 4 months and altered coagulation values consisting of increased
9   fibrinogen in males at both times and shortened prothrombin time in females at 6 months (data
10 not shown). No treatment related effects were detected on the types of white cells in peripheral
11 blood." It must be noted that effects reported from this study may have also been related to
12 decreased water consumption, this study did included any light microscopic evaluation, and that
13 most of the results described are for data not shown. However, this study does illustrate the
14 difficulties involved in trying to conduct studies of TCE in drinking water, that the LD50s for
15 TCE are relatively high, and that liver weight increases were observed with TCE exposure as
16 early as few weeks and increased liver weight were sustained through the 6 month study period.
17
18 **2.1.7. Goldsworthy and Popp 1987**
19
20   The focus of this study was peroxisomal proliferation activity after exposure to a number
21 of chlorinated solvents. In this study 1000 mg/kg TCE (99+ % epoxide stabilizer free) was
22 administered to male F-344 rats (170-200 g or ~ 10% difference) and B6C3F1 (20-25 g or ~ 20%
23 difference) mice for 10 days in corn oil via gavage. The ages of the animals were not given. The
24 TCE-exposed animals were studied in two experiments (Experiments #1 and #3). In experiment
25 #2 corn oil and methyl cellulose vehicles were compared. Animals were killed 24 hours after the
26 last exposure. The authors did not show data on body weight but stated that the administration of
27 test agents (except WY-14,643 to rats which demonstrated no body weight gain) to rats and mice
28 for 10 days "had little or no effect on body weight gain." Thus, differences in initial body
29 weight between treatment and control groups, which could have affected the magnitude of TCE-
30 induced liver weight gain, were not reported. The liver/body weight ratios in corn oil gavaged
31 rats were reported to be $3.68 \pm 0.06\%$ and $4.52 \pm 0.08\%$ after TCE treatment which represented
32 1.22-fold of control (n = 5). CN-insenstive palmitoyl CoA oxidation (PCO) was reported to be
33 1.8-fold increased after TCE treatment in this same group. In B6C3F1 mice the liver/body
34 weight ratio in corn oil gavaged mice was reported to be $4.55 \pm 0.13\%$ and $6.83 \pm 0.13\%$ after
35 TCE treatment which represented 1.50-fold of control (n = 7). CN-insensitive PCO activity was
36 reported to be 6.25-fold of control after TCE treatment in this same group. The authors report no
37 effect of vehicle on PCO activity but do not show the data nor discuss any effects of vehicle on
38 liver weight gain. Similarly the results for experiment #3 were not shown nor liver weight
39 discussed with the exception of PCO activity reported to be 2.39-fold of control in rat liver and
40 6.25-fold of control for mouse liver after TCE exposure. The number of animals examined in
41 experiment #3 was not given by the authors or the variation between enzyme activities.
42 However, there appeared to be a difference in PCO activity experiments #1 and #3 in rats.

1   There were no descriptions of liver histology given in this report for TCE-exposed animals or
2   corn-oil controls.
3
4   **2.1.8.   Elcombe et al. 1985**
5
6   In this study, preservative free TCE was given via gavage to rats and mice for 10
7   consecutive days with a focus on changes in liver weight, structure, and hepatocellular
8   proliferation induced by TCE. Male Alderly Park rats (Wistar derived) (180-230 g), male
9   Obsborne-Mendel rats (240-280 g), and male B6C3F1 or male Alderly Park Mice (Swiss)
10 weighing 30 to 35 g were administered 99.9% pure TCE dissolved in corn oil via gavage. The
11 ages of the animals were not given by the authors. The animals were exposed to 0, 500, 1000 or
12 1500 mg/kg body wt TCE for 10 consecutive days. The number of mice and rats varied widely
13 between experiments and treatment groups and between various analyses.   In some experiments
14 animals were injected with tritiated thymidine approximately 24 hours following the final dose
15 of TCE and killed one hour later. The number of hepatocytes undergoing mitosis was identified
16 in 25 random high-power fields (X40) for each animal with 5000 hepatocyte per animal
17 examined. There was no indication by the authors that zonal differences in mitotic index were
18 analyzed. Sections of the liver were examined by light and electron microscopy by conventional
19 staining techniques. Tissues selected for electron microscopy included central vein and portal
20 tract so that zonal differences could be elucidated. Morphometric analysis of peroxisomes was
21 performed "according to general principles of Weibel et al (1964) on electronphotomicrographs
22 from pericentral hepatocytes." DNA content of samples and peroxisomal enzyme activities were
23 determined in homogenized liver (catalase and PCO activity).
24
25   The authors reported that TCE treatment had no significant effect on body weight gain
26 either strain of rat or mouse during the 10 days exposure period.   However, marked increases
27 (up to 175% of control value) in the % liver/body weight ratio were observed in TCE treated
28 mice. Smaller increases (up to 130% of control) in relative liver weight were observed in TCE-
29 treated rats. No significant effects of TCE on hepatic water content were seen so that the liver
30 weight did not represent increased water retention.
31
32   An interesting feature of this study was that it was conducted in treatment blocks at
33 separate times with separate control groups of mice for each experimental block. Therefore,
34 there were 3 control groups of B6C3F1 mice (n= 10 for each control group) and 3 control groups
35 for Alderly Park (n= 9 to 10 for each control group) mice that were studied concurrently with
36 each TCE treatment group. However, the % liver/body weight ratios were not the same between
37 the respective control groups. There was no indication from the authors as to how controls were
38 selected or matched with their respective experimental groups. The authors did not give liver
39 weights for the animals so the actual changes in liver weights are not given. The body weights
40 of the control and treated animals were also not given by the authors. Therefore, if there were
41 differences in body weight between the control groups or treatment groups, the liver/body weight
42 ratios could also have been affected by such differences. The percentage increase over control
43 could also have been affected by what control group each treatment group was compared to.

1   There was a difference in the mean % liver/body weight ratio in the control groups which ranged
2   from 4.32 to 4.59% in the B6C3F1 mice (~ 6% difference) and from 5.12 to 5.44 % in the
3   Alderly Park mice (~ 6% difference). The difference in average % liver/body weight ratio for
4   untreated mice between the two strains was ~ 16%. Because the ages of the mice were not
5   given, the apparent differences between strains may have been due to both age or to strain. After
6   TCE exposure, the mean % liver/body weight ratios were reported to be 5.53% for 500 mg/kg,
7   6.50% for 1000 mg/kg and 6.74% for 1500 mg/kg TCE exposed B6C3F1 mice. This resulted in
8   1.20-fold, 1.50-fold and 1.47-fold values of control in % liver weight/body weight for B6C3F1
9   mice. For Alderly Park mice the % liver/body weight ratios were reported to be 7.31, 8.50 and
10 9.54 % for 500, 1000, and 1500 mg/kg TCE treatment, respectively. This resulted in 1.43-fold,
11 1.56-fold and 1.75-fold of control values. Thus, there appeared to be more of a consistent dose-
12 related increase in liver/body weight ratios in the Alderly Park mice than the B6C3F1 mice after
13 TCE treatment. However, the variability in control values may have distorted the dose-response
14 relationship in the B6C3F1 mice. The Standard deviations for liver/body weight ratio were as
15 much as 0.52 % for the treated B6C3F1 mice and 0.91% for the Alderly Park treated mice.   In
16 regard to the correspondence of the magnitude of the TCE-induced increases in % liver/body
17 weight with the magnitude of difference in TCE exposure concentrations, in the B6C3F1 mice
18 the increases were similar (~ 2-fold) between the 500 mg/kg and 1000 mg/k TCE exposure
19 groups. For the Alderly Park mice, the increases in TCE exposure concentrations were slightly
20 less than the magnitude of increases in % liver/body ratios between all of the concentrations (i.e.,
21 ~1.3 fold of control vs. 2-fold for 500 and 1000 mg/kg TCE dose and 1.3-fold of control vs. 1.5-
22 fold for the 1000 and 1500 mg/kg TCE dose).
23
24   The DNA content of the liver varied greatly between control animal groups. For B6C3F1
25 mice it ranged from 2.71 to 2.91 mg/g liver. For Alderly Park mice it ranged from 1.57 to 2.76
26 mg/g liver. The authors do not discuss this large variability in baseline levels of DNA content.
27 The DNA content in B6C3F1 mice was mildly depressed by TCE treatment in a non-dose
28 dependent manner. DNA concentration decrease from control ranged from 20-25% between all
29 three TCE exposure levels in B6C3F1 mice. For Alderly Park mice there was also non-dose
30 related decrease in DNA content from controls that ranged from 34% to 18%. Thus, the extent
31 of decrease in DNA content of the liver from TCE treatment in B6C3F1 mice was similar to the
32 variability between control groups. The lack of dose-response in apparent treatment related
33 effect in B6C3F1 mice and especially in the Alderly Park mice was confounded by the large
34 variability in the control animals. The changes in liver weight after TCE exposure for the AP
35 mice did not correlate with changes in DNA content further, raising doubt about the validity of
36 the DNA content measures. However, a small difference in DNA content due to TCE treatment
37 in all groups was reported for both strains and this is consistent with hepatocellular hypertrophy.
38
39   The reported results for incorporation of tritiated thymidine in liver DNA showed large
40 variation in control groups and standard deviations that were especially evident in the Alderly
41 Park mice. For B6C3F1 mice, mean control levels were reported to range from 5,559 to 7,767
42 dpm/mg DNA with standard deviations ranging from 1,268 to 1,645 dpm/mg DNA. In Alderly
43 Park mice mean control levels were reported to range from 6,680 to 10,460 dpm/mg DNA with

1 standard deviations ranging from 308 to 5,235 dpm/mg DNA. For B6C3F1 mice, TCE treatment
2 was reported to induce an increase in tritiated thymidine incorporation with a very large standard
3 deviation, indicating large variation between animals. For 500 mg/kg TCE treatment group the
4 values were reported as 12,334 ± 4,038, for 1000 mg/kg TCE treatment group 21,909 ± 13,386,
5 and for 1500 mg/kg treatment TCE group 26,583 ± 10,797 dpm/mg DNA. In Alderly Park mice
6 TCE treatment was reported to give an increase in tritiated thymidine incorporation also with a
7 very large standard deviation. For 500 mg/kg TCE the values were reported as 19,315 ± 12,280,
8 for 1000 mg/kg TCE 21,197 ± 8,126 and for 1500 mg/kg TCE 38,370 ± 13,961. As a percentage
9 of concurrent control, the increase in tritiated thymidine was reported to be 2.11-fold, 2.82-fold,
10 and 4.78-fold of control in B6C3F1 mice, and 2.09-fold, 2.03-fold, and 5.74-fold of control in
11 Alderly Park mice. Accordingly, the change in tritiated thymidine incorporation did show a
12 treatment related increase but not a dose-response. Similar to the DNA content of the liver, the
13 large variability in measurements between control groups and variability between animals limit
14 quantitative interpretation of this data. The increase in tritiated thymidine, seen most
15 consistently only at the highest exposure level in both strains of mice, could have resulted from
16 either a change in ploidy of the hepatocytes or cell number. However, the large change in
17 volume in the liver (75%) in the Alderly Park mice, could not have resulted from only a 4-fold of
18 control in cell proliferation even if all tritiated thymidine incorporation had resulted from
19 changes in hepatocellular proliferation. As mentioned in Section 1.1 above, the baseline level of
20 hepatocellular proliferation in mature control mice is very low and represents a very small
21 percentage of hepatocytes.
22
23   In the experiments with male rats, the same issues discussed above, associated with the
24 experimental design, applied to the rat experiments with the additional concern that the numbers
25 of animals examined varied greatly (i.e., 6 to 10) between the treatment groups. In Obsborne-
26 Mendel rats the control liver/body weight ratio was reported to vary from 4.26 to 4.36% with the
27 standard deviations varying between 0.22 to 0.27%. For the Alderly Park rats the liver/body
28 weight ratios were reported to vary between 4.76 and 4.96% (in control groups with standard
29 deviations varying between 0.24 to 0.47%. TCE treatment was reported to induce a dose-related
30 increase in liver/body weight ratio in Obsborne-Mendel rats with mean values of 5.16, 5.35 and
31 5.53% in 500, 1000 and 1500 mg/kg TCE treated groups, respectively. This resulted in 1.18-
32 fold, 1.26-fold, and 1.30-fold values of control. In Alderly Park rats, TCE treatment was
33 reported to resulted increased liver weights of 5.45, 5.83 and 5.65 % for 500, 1000, and 1500
34 mg/kg TCE respectively. This resulted in 1.14-fold, 1.17-fold, and 1.17-fold values of control.
35 Again, the variability in control values may have distorted the nature of the dose-response
36 relationships in Alderly Park rats. TCE treatment was reported to result in standard deviations
37 that ranged from 0.31 to 0.48% for OM rats and 0.24 to 0.38% for Alderly Park rats. What is
38 clear from these experiments is that TCE exposure was associated with increased liver/body
39 weight in rats.
40
41   The reported mean hepatic DNA concentrations and standard deviations varied greatly in
42 control rat liver as it did in mice. The variation in DNA concentration in the liver varied more
43 between control groups than the changes induced by TCE treatment. For Obsborne-Mendel rats

the mean control levels of mg DNA/g liver were reported to range from 1.99 to 2.63 mg DNA/liver with standard deviations varying from 0.17 to 0.33 mg DNA/g. For Alderly Park rats the mean control levels of mg DNA/ g liver were reported to be 2.12 to 3.16 mg DNA/g with standard deviation ranging from 0.06 to 1.04 mg DNA/g. TCE treatment decreased the liver DNA concentration in all treatment groups. For Obsborne-Mendel rats the decrease ranged from 8 to 13% from concurrent control values and for Alderly Park rats the decrease ranged from 8 to 17%. There was no apparent dose-response in the decreases in DNA content with all TCE treatment levels giving a similar decrease from controls and the same limitations discussed above for the mouse data apply here. The magnitude of increases in liver/body ratios shown by TCE treatment were not correlated with the changes in DNA content. However, as with the mouse data, the small differences in DNA content due to TCE treatment in all groups and in both strains was consistent with hepatocellular hypertrophy.

Incorporation of tritiated thymidine was reported to be even more variable between control groups of rats than it was for mice and was reported to be especially variable between control groups (i.e., 2.7-fold difference between control groups within strain) and differed between the strains (average of 2.5 fold between strains). For Obsborne-Mendel rats the mean control levels were reported to range from 13,315 to 33,125 dpm/mg DNA, while for Alderly Park rats tritiated thymidine incorporation ranged from 26,613 to 69,331 dpm/mg DNA for controls. The standard deviations were also very large (i.e., for control groups of Obsborne-Mendel rats they were reported to range from 8,159 to 13,581 dpm/mg DNA, while for Alderly Park rats they ranged from 9,992 to 45,789 dpm/mg DNA). TCE treatment was reported to induce increases over controls of 110, 118, and 106% for 500, 1000, and 1500 mg/kg TCE exposed groups, respectively, in Obsborne-Mendel rats with large standard deviations for these treatment groups as well. In Alderly Park rats the increases over controls were reported to be 206, 140 and 105% for 500, 1000, and 1500 mg/kg TCE, respectively. In general, these data do indicate that TCE treatment appeared to give a mild increase in tritiated thymidine incorporation but the lack of dose-response can be attributable to the highly variable measurements of tritiated thymidine incorporation in control animal groups. The variation in the number of animals examined between groups and small numbers of animals examined additionally decrease the likelihood of being able to discern the magnitude of difference between species- or strain-related effects for this parameter. Again, given the very low level of hepatocyte turnover in control rats, this does not represent a large population of cells in the liver that may be undergoing proliferation and cannot be separated from changes in ploidy.

The authors report that the reversibility of these phenomena was examined after the administration of TCE to Alderly Park mice for 10 consecutive days. Effects upon liver weight, DNA concentration, and tritiated thymidine incorporation 24 and 48 hours after the last dose of TCE were reported to still be apparent. However, 6 days following the last dose of TCE, all of these parameters were reported to return to control values with the authors not showing the data to support this assertion. Thus, cessation of TCE exposure would have resulted in a 75% reduction in liver weight by one week in mice exposed to the highest TCE concentration.

1   Analyses of hepatic peroxisomal enzyme activities were reported for catalase and β-
2   oxidation (PCO activity) following administration of TCE to B6C3F1 mice and Alderly Park rats
3   exposed to 1000 mg/kg TCE for 10 days. The authors only used 5 control and 5 exposed
4   animals for these tests. An 8-fold of control value for PCO activity and a 1.5-fold of control
5   value for catalase activity were reported for B6C3F1 mice exposed to 1000 mg/kg TCE. In the
6   Alderly Park rats no significant changed occurred. It is unclear which mice or rats were selected
7   from the previous experiments for these analyses and what role selection bias may have played
8   in these results. The reduced number of animals chosen for this analysis also reduces the power
9   of the analysis to detect a change. In rats there was a reported 13% increase in PCO, however
10   the variation between the TCE treated rats was more than double that of the control animals in
11   this group and the other limitations described above limit the ability to detect a response. There
12   was no discussion given by the authors as to why only one dose was tested in half of the animals
13   exposed to TCE or why the strain with the lowest liver weight change due to TCE exposure was
14   chosen as the strain to test for peroxisomal proliferative activity.
15
16   The authors provided a description of the histopathology at the light microscropy level in
17   B6C3F1 mice, Alderly Park mice, Osborne-Mendel rats, and Alderly Park rats, but did not
18   provide a quantitative analysis or specific information regarding the variability of response
19   between animals within groups. There appeared to be 20 animals examined in the 1000 mg/kg
20   TCE exposed group of B6C3F1 mice but no explanation as to why there were only 10 animals
21   examined in analyses for liver weight changes, DNA concentration and tritiated thymidine
22   incorporation. There was no indication by the authors regarding how many rats were examined
23   by light microscopy.
24
25   Apart from a few inflammatory foci in occasional animals, H&E section from B6C3F1
26   control mice were reported to show no abnormalities. The authors suggest that this is a normal
27   finding in the livers of mice kept under "non-SPF conditions". A stain for neutral lipid was
28   reported to not be included routinely in these studies, but subsequent electron microscopic
29   examination of lipid to show increases in the livers of corn-oil treated control animals. The
30   individual fat droplets were described as "generally extremely fine and are not therefore
31   detectable in conventionally process H&E stained sections, since both glycogen and lipid are
32   removed during this procedure." Thus, this study documents effects of using corn oil gavage in
33   background levels of lipid accumulation in the liver.
34
35   The finding of little evidence of gross hepatotoxicity in TCE-treated mice was reported,
36   even at a dose of 1500 mg/kg. Specifically, "Of 19 animals examined receiving 1500 mg/kg
37   body weight TCE, only 6 showed any evidence of hepatocyte necrosis, and this pathology was
38   restricted to single small foci or isolated single cells, frequently occurring in a subcapsular
39   location. Examination of 20 animals receiving 1000 mg/kg body wt TCE demonstrated no
40   hepatocyte necrosis. Of 20 animals examined receiving 500 mg/kg body wt TCE, 1 showed
41   necrosis of single isolated hepatocytes; however, this change was not a treatment-related
42   finding." TCE-treated mice were reported to show "a change in staining characteristic of the
43   hepatocytes immediately adjacent to the central vein of the hepatocyte lobules, giving rise to a

1 marked 'patchiness' of the liver sections. Often this change consisted of increased eosinophilia
2 of the central cells. There was some evidence of cell hypertrophy in the centrilobular regions.
3 These changes were evident in most of the TCE treated animals, but there was a dose-related
4 trend, relatively few of the 500 mg/kg animals being affected, while the majority of the 1500
5 mg/kg animals showed central change. No other significant abnormalities were seen in the liver
6 of TCE treated mice compared to controls apart from occasional mitotic figures and the
7 appearance of isolated nuclei with an unusual chromatin pattern. This pattern generally
8 consisted of a course granular appearance with a prominent rim of chromatin around the
9 periphery of the nucleus. These nuclei may have been in the very early stages of mitosis.
10 Similar changes were not seen in control mice." The authors briefly commented on the findings
11 in the Alderly Park mice stating that "H& E sections from Alderly Park mice gave similar results
12 as for B6C3F1 mice. No evidence of hepatotoxicity was seen at a dose of 500 mg/kg body wt
13 TCE. However, a few animals at the higher doses showed some necrosis and other degenerative
14 changes. This change was very mild in nature, being restricted to isolated necrotic cells or small
15 foci, frequently in subcapsular position. Hypertrophy and increased eosinophilia were also
16 noticed in the centrilobular regions at higher doses." Thus, from the brief description given by
17 the authors, the centrilobular region is identified as the location of hepatocellular hypertrophy
18 due to TCE exposure in mice, and for it to be dose-related with little evidence of accompanying
19 hepatotoxicity.
20
21   The description of histopathology for rats was even more abbreviated than for the mouse.
22 H& E sections from Osborne-Mendel rats showed that "livers from control rats contained large
23 quantities of glycogen and isolated inflammatory foci, but were otherwise normal. The majority
24 of rats receiving 1500 mg/kg body weight TCE showed slight changes in centrilobular
25 hepatocytes. The hepatocytes were more eosinophilic and contained little glycogen. At lower
26 doses these effects were less marked and were restricted to fewer animals. No evidence of
27 treatment-related hepatotoxicity (as exemplified by single cell or focal necrosis) was seen in any
28 rat receiving TCE. H& E sections from Alderly Park Rats showed no signs of treatment-related
29 hepatotoxicity after administration of TCE. However, some signs of dose-related increase in
30 centrilobular eosinophilia were noted." Thus both mice and rats exhibited pericentral
31 hypertrophy and eosinophilia as noted from the histopathological examination.
32
33   The study did report a quantitative analysis of the effects of TCE on the number of
34 mitotic figures in livers of mice. Few if any control mice exhibited mitotic figures. But the
35 authors report "a considerable increase in both the numbers of figures per section was noted after
36 administration of TCE". The numbers of animals examined for mitotic figures ranged from 75
37 (all control groups were pooled for mice) to 9 in mice, and ranged from 15 animals in control rat
38 groups to as low as 5 animals in the TCE treatment groups. The range of mitotic figures found in
39 25 high-power fields was reported and is equivalent to the number of mitotic figures per 5000
40 hepatocytes examined in random fields".   Thus the predominance of mitotic figures in any zone
41 of the liver cannot be ascertained.

1   For B6C3F1 mice the number of animals with mitotic figures was reported to be 0/75,
2   3/20, 7/20 and 5/20 for control, 500, 1000, and 1500 mg/kg TCE exposed mice, respectively.
3   The range of the number of mitotic figures seen in 5000 hepatocytes was reported to be 0, 0-1, 0-
4   5, 0-5 for those same groups with group means of 0, 0.15 ± 0.36, 0.6 ± 1.1 and 0.5 ± 1.2. These
5   results demonstrate a very small and highly variable response due to TCE treatment in B6C3F1
6   mice in regard to mitosis. Thus, the highest percentage of cells undergoing mitosis within the
7   window of observation would be on average 0.012% with a standard deviation twice that value.
8   The data presented for mitotic figures also indicated no differences in results between 1000 and
9   1500 mg/kg treated B6C3F1 mice in regard to mitotic figure detection. However, the tritiated
10   thymidine incorporation data indicated that thymidine incorporation was ~2-fold greater at 1500
11   than 1000 mg/kg TCE in B6C3F1 mice. For Alderly Park mice, the number of animals with
12   mitotic figures was reported to be 1/15, 0/9, 4/9 and 2/9 for control, 500, 1000, and 1500 mg/kg
13   TCE exposed mice. The range of the number of mitotic figures seen in 5000 hepatocytes was 0-
14   1, 0, 0-2, 0-1 for those same groups with group means of 0.06 ± 0.25, 0. 0.7 ± 0.9, and 0.2 ± 0.4.
15   These results reveal the detection of at the most 2 mitotic figure in 5000 hepatocytes for any
16   mouse an any treatment group and no dose-related increased after TCE treatment in Alderly Park
17   mice. Thus, the highest percentage of cells with a mitotic figure would be on average 0.014%
18   with a standard deviation twice that value. The small number of animals examined reduces the
19   power of the experiment to draw any conclusions as to a dose-response. Similar to the B6C3F1
20   mice, there did not appear to be concordance between mitotic figure detection and thymidine
21   incorporation for Alderly park mice. Thymidine incorporation showed a 2- fold increase over
22   control for 500 and 1000 mg/kg TCE and a 5.7- fold increase for 1500 mg/kg TCE treated
23   animals. However, in regard to mitotic figure detection, there were fewer mitotic figures in 500
24   mg/kg TCE treated mice than controls, and fewer animals with mitotic figures and fewer
25   numbers of figures in the 1500 mg/kg dose than the 1000 mg/kg exposed group. The
26   inconsistencies between mitotic index data and thymidine incorporation data in both strains of
27   mice suggests that either thymidine incorporation is representative of only DNA synthesis and
28   not mitosis, an indication of changes in ploidy rather than proliferation, or that this experimental
29   design is incapable of discerning the magnitude of these changes accurately. Data from both
30   mouse strains show very little if any hepatocyte proliferation due to TCE exposure with the
31   mitotic figure index data having that advantage of being specific for hepatocytes and to not to
32   also include nonparenchymal cells or inflammatory cells in the liver.
33
34   The results for rats were similar to those for mice and even more limited by the varying
35   and low number of animals examined. For Osborne-Mendal rats the number of animals with
36   mitotic figures were reported to be 8/15, 2/9, 0/7 and 0/6 for control, 500, 1000, and 1500 mg/kg
37   TCE exposed rats groups, respectively, with the range of the number of mitotic figures seen in
38   5000 hepatocytes to be 0-8, 0-3, 0, and 0. The group mean was 1.5 ± 2.0, 0.4 ± 1.0, 0 and 0 for
39   these groups. It would appear from these results that there are fewer mitotic figures after TCE
40   treatment with the highest percentage of cells undergoing mitosis to be on average 0.03% in
41   control rats. However, thymidine incorporation studies show a modest increase at all treatment
42   levels over controls in Osborne Mendel rats rather than a decrease from controls. For Alderly
43   Park rats the number of animals with mitotic figures was reported to be 13/15, 5/9, 9/9 and 4/9

1   for control, 500, 1000, and 1500 mg/kg TCE exposed rat groups with the range of the number of
2   mitotic figures seen in 5000 hepatocytes to be 0-26, 0-5, 1-7, and 0-9. The group mean was 7.2
3   ± 4.7, 1.6 ± 4.3, 3.8 ± 3.4 and 1.8 ± 2.9 for these groups. It would appear that there are fewer
4   mitotic figures after TCE treatment with the highest percentage of cells to an average of 0.14%
5   in control rats. However, thymidine incorporation studies show 2-fold greater level at 500 mg/kg
6   TCE than for control animals and a 40% and 5% increase at 1000 mg/kg and 1500 mg/kg TCE
7   exposure groups, respectively.   Similar to the results reported in mice, results in both rat strains
8   show an inconsistency in mitotic index and thymidine incorporation.   The control rats appear to
9   have a much greater mitotic index than any of the mouse groups (treated or untreated) or the
10   TCE treatment groups. However, it is the mice that were exhibiting the largest increased in liver
11   weight after TCE exposure. By either thymidine incorporation or mitosis, these data do provide
12   a consistent result that at 10 days of exposure very little sustained hepatocellular proliferation is
13   occurring in either mouse or rat and neither is correlated well with the concurrent changes in
14   liver weight observed from TCE exposure.

15

16   This study provided a qualitative discussion and quantitative analysis of structural
17   changes using electron microscopy. The qualitative discussion was limited and included
18   statements about increased observances without quantitative data shown other than the
19   morphometric analysis. The authors reported that "the ultrastructure of control mouse liver was
20   essentially normal, although mild dilatation of RER and SER was a frequent finding. Lipid
21   droplets were also usually present in the cell cytoplasm. The ultrastructural changes seen in
22   mouse liver following administration of up to 1500 mg/kg body wt TCE for 10 days were
23   essentially similar in the B6C3F1 mouse and the Alderly Park mouse. The most notable change
24   in both strains of mouse was a dramatic increase in the number of peroxisomes. This change was
25   only apparent in the cells immediately surrounding the central veins. Peroxisome proliferation
26   was not noticeable in periportal cells. The induced peroxisomes were generally small and very
27   electron dense and frequently lacked the characteristic nucleoid core found in peroxisomes of
28   control livers." The authors conclude that "morphometric analysis showed evidence of a dose-
29   related response, peroxisomal induction appearing to reach a maximum at 1000 mg/kg in
30   B6C3F1 mice." "Lipid was increased in the livers of treated mice at all doses and was present
31   both as free droplets in the cytoplasm and as liposomes (small lipid droplets in ER cisternae).
32   The centrilobular cell which showed the greatest increase in numbers of peroxisomes showed no
33   evidence of this lipid accumulation: fatty change was more prominent in those cells away from
34   the central vein (i.e., zone 2 of the liver acinus). Accumulation of lipid, particularly in
35   liposomes, was less marked in Alderly Park mouse than in B6C3F1 mouse. Mild proliferation of
36   smooth endoplasmic reticulum was seen in both strains and both rough and smooth endoplasmic
37   reticulum was generally more dilated than in control mice." Electron microscopic results for rat
38   liver were reported "to show similar changes in Osborne-Mendel and Alderly Park rat treated
39   with TCE." "Rats receiving either 1000 or 1500 mg/kg TCE for 10 days generally showed mild
40   proliferation of SER in centrilobular hepatocytes. The cisternae of RER were frequently dilated,
41   giving rise to a rather disorganized appearance in contrast to the parallel stacks seen in control
42   livers, although no detachment of ribosomes was evident. The SER was also dilated. In contrast
43   to mice, peroxisomes were only very slightly and not significantly, increased in the liver of TCE

1 –treated rats. Morphometric analysis confirmed this observation, with the volume density of
2 peroxisomes in the cytoplasm of centrilobular hepatocytes being only slightly increased in rats of
3 both strains receiving 1000 or 1500 mg/kg body wt TCE."   "Lipid droplets were occasionally
4 increased in some livers obtained from rats receiving TCE, but the degree of fatty change
5 generally appeared similar to that found in control rats receiving corn oil. There were no
6 changes in membrane –bound liposomes, other organelles, or Golgi condensing vesicles.
7 Centrilobular glycogen was somewhat depleted in male rats receiving 1500 mg/kg TCE.
8 Periportal cells were ultrstructually normal in all rats."
9
10   For the morphometric analysis, the number of mice examined ranged from 7 in the
11 control group to 8 in the 1500 mg/kg TCE exposed group. The authors did not indicate which
12 control animals were used for the morphometric analysis from the 75 animals examined for
13 mitotic index, the 20 examined by light microscopy, or the 30 mice used as concurrent controls
14 in the liver weight, DNA concentration and tritiated thymidine incorporation studies. The
15 authors stated that morphometry was performed on three randomly selected photomicrographs
16 from each of three randomly selected pericentral hepatocytes for each animal (i.e., nine
17 photomicrographs per animal). A mean value representing the exposure group was reported with
18 the variability between photomitographs per animal or the variation between animals unclear.
19 The morphometric analysis did not examine all treatment groups (e.g., only the control and 500
20 mg/kg TCE group were examined in Alderly Park mice). The % cytoplasmic volume of the
21 peroxisomal compartment (x ± SD) was reported to be 0.6 ± 0.6 % for controls, 4.8 ± 3.3 % for
22 500 mg/kg TCE, 6.7 ± 1.9 % for 1000 mg/kg TCE, and 6.4 ± 2.5% for 1500 mg/kg TCE in
23 B6C3F1 mice.   In Alderly Park mice, only 12 control and 12 500 mg/kg TCE exposed mice
24 were examined and, similarly, their selection criteria was not given. The % cytoplasmic volume
25 of the peroxisomal compartment was 1.2 ± 0.4% for control and 4.7 ± 2.8 % for 500 mg/kg TCE
26 exposed mice.   For Osborne-Mendel rats control rats were reported to have a % cytoplasmic
27 volume of the peroxisomal compartment for control rats (n=9) of 1.8 ± 0.4 %, 1000 mg/kg TCE
28 (n= 5) 2.3 ± 1.6 %, and for 1500 mg/kg exposed rats (n= 7) 2.3 ± 2.0%. For Alderly Park rats
29 only two groups were examined (control and 1000 mg/kg TCE exposure). The % cytoplasmic
30 volume of the peroxisomal compartment for control rats (n= 15) was reported to be 1.8 ± 0.8 %
31 and for 1000 mg/kg TCE (n= 16) to be 2.4 ± 1.2 %. The varying numbers of animals examined,
32 the varying and inconsistent number of treatment groups examined, the limited number of
33 photomitographs per animal, and the potential selection bias for animals examined make
34 quantitative conclusions regarding this analysis difficult. Although control levels differed by a
35 factor of 2 between the two strains of mice examined, as well as the number of control animals
36 examined (7 vs. 12), it appears that the 500 mg/kg TCE exposed B6C3F1 and Alderly Park mice
37 had similar percentages of peroxisomal compartment in the pericentral cells examined (~ 4.8 %).
38 There also appeared to be little difference between 1000 mg/kg TCE treated Osborne-Mendel
39 and Alderly Park rats for this parameter (~2.4%). Although few animals were examined, there
40 was little difference reported between 500, 1000 and 1500 mg/kg TCE exposure groups in regard
41 to percentages of peroxisomal compartment in B6C3F1 mice (4.8 – 6.7%). For the few rats of
42 the Osborne-Mendel strain examined, there also did not appear to be a difference between 1000
43 and 1500 mg/kg TCE exposure for this parameter (2.3%).

1   Based on peroxisome compartment volume data, one would expect there to be little
2   difference between TCE exposure groups in mice or rats in regard to enzyme activity or other
3   "associated events." However such comparisons are difficult due to limited power to detect
4   differences and the possibility of bias in selection of animals in differing assays.   For the
5   B6C3F1 mice, only 5 animals per group were examined for enzyme analysis, 7 to 8 for
6   morphometric analysis, 75 animals in control and 20 animals in 1000 mg/kg TCE exposed
7   groups for mitotic figure identification, and 10 animals per group for thymidine incorporation.
8   Since only a few animals were tested for enzyme activity the comparison between peroxisomal
9   compartment volume and that parameter is very limited. There was a reported 47% increase in
10   catalase activity between control (n = 5) and 1000 mg/kg TCE exposed B6C3F1 mice (n = 5) and
11   7.8- fold increase in PCO activity. The % peroxisome compartment was reported to be 10.6-fold
12   greater (0.6 vs. 6.4%). However, the B6C3F1 control % volume of peroxisomal compartment
13   was reported to be half that of the AP mouse control. An accurate determination of the
14   quantitative differences in peroxisomal proliferation would be dependent on an accurate and
15   stable control value. For Alderly Park rats there was an 8% decrease in catalase activity between
16   control (n = 5) and 1000 mg/kg TCE exposed rats (n = 5), and a 13% increase in PCO activity.
17   The % peroxisome compartment was reported to be 33% greater in the TCE-exposed than
18   control group. Thus, for the very limited data that was available to compare peroxisomal
19   compartment volume with enzyme activity, there was consistency in result.
20
21   However, were such increases in peroxisomes associated with other events reported in
22   this study? Mouse peroxisome proliferation associated enzyme activities in B6C3F1 mice at
23   1000 mg/kg TCE were reported to be 8-fold over control values in mice after 10 days of
24   treatment. However, this increase in activity was not accompanied by a similar increase in
25   thymidine incorporation (2.8- fold of control) or concordant with increases in mitotic figures
26   (7/20 mice having any mitotic figures at all with a range of 0 – 5 and a mean of 0.014% of cells
27   undergoing mitosis for 1000 mg/kg TCE vs. 0 for control). Although results reported in the rat
28   showed discordance between thymidine incorporation and detection of mitotic figures, there was
29   also discordance with these indices and those for peroxisomal proliferation.   In comparison to
30   controls, there was a reported 13% increase in PCO activity in Alderly park rats exposed to 1000
31   mg/kg TCE, a group mean of mitotic figures half that in the TCE treated animals vs. controls,
32   and increase in thymidine incorporation of 40%. Thus, these results are not consistent with TCE
33   induction of peroxisome enzyme activity to be correlated with hepatocellular proliferation by
34   either mitotic index or thymidine incorporation. Thymidine incorporation in liver DNA seen
35   with TCE exposure also did not correlate with mitotic index activity in hepatocytes and suggests
36   that this parameter may be a reflection of polyploidization rather than hepatocyte proliferation.
37   More importantly, these data show that hepatocyte proliferation, indicated by either measure, is
38   confined to a very small population of cells in the liver after 10 days of TCE exposure.
39   Hepatocellular hypertrophy in the centrilobular region appears to be responsible for the liver
40   weight gains seen in both rats and mice rather than increases in cell number. These results at 10
41   days do not preclude the possibility that a greater level of hepatocyte proliferation did not occur
42   earlier and then had subsided by 10 days, as is characteristic of many mitogens. Thymidine
43   incorporation represents the status of the liver at one time point rather than over a period of

1   whole week and thus would not capture the earlier bouts of proliferation. However, there is no
2   evidence of a sustained proliferative response, as measured at the 10-day time period, in
3   hepatocytes in response to TCE indicated from this data.
4
5   In regards to weight gain, although the volume of the peroxisomal compartment was
6   reported to be similar at 500 mg/kg TCE in B6C3F1 and Alderly Park mice (4.3%), the liver
7   weight./body weight gain in comparison to control was 20% higher in B6C3F1 mice vs. 43%
8   higher in Alderly Park mice after 10 days of exposure. The liver/body weight ratio was 5.53 %
9   in the B6C3F1 mice and 7.31% in the Alderly Park mice at 500 mg/kg TCE for 10 days.
10 Similarly, although the peroxisomal compartment was similar at 1000 mg/kg TCE in Osborne-
11 Mendel (2.3%) and Alderly Park rats (2.4%), the liver weight/body weight gain was 26% in
12 Osborne-Mendel rats but 17% in Alderly Park rats at this level of TCE exposure. The liver/body
13 weight ratio was 5.35 % in the Osborne-Mendel rats and 5.83% in the Alderly Park mice at 1000
14 mg/kg TCE for 10 days. Although there are several limitations regarding the quantitative
15 interpretation of the data, as discussed above, the data suggest that liver weight and weight gain
16 after TCE treatment was not just a function of peroxisome proliferation. This study does clearly
17 demonstrate TCE-induced changes at the lowest level tested in several parameters without
18 toxicity and without evidence of regenerative hyperplasia or sustained hepatocellular
19 proliferation. In regards to susceptibility to liver cancer induction in more susceptible (B6C3F1)
20 vs. less susceptible (Alderly Park/Swiss) strains of mice (Maltoni et al., 1988), there was a
21 greater baseline level of liver weight/body weight ratio change, a greater baseline level of
22 thymidine incorporation as well as greater responses for those endpoints due to TCE exposure in
23 the "less susceptible" strain. However, both strains showed a hepatocarcinogenic response to
24 TCE induction and the limitations of being able to make quantitative conclusions regarding
25 species and strain susceptibility TCE toxicity from this study have been described in detail
26 above.
27
28 **2.1.9. Dees and Travis 1993**
29
30   The focus of this study was to evaluate the nature of DNA synthesis induced by TCE
31 exposure in mice. The mitotic rate of liver cells was extrapolated using tritiated thymidine
32 uptake into DNA of male and female mice treated with HPLC grade (99+pure) TCE. Male and
33 female hybrid B6C3F1 mice 8 weeks of age (male mice weighed 24-27 g (~ 12% difference)
34 and females weighing 18-21 g (~ 14% difference) were dosed orally by gavage for 10 days with
35 100, 250, 500, and 1000 mg/kg body weight TCE in corn oil (n=4 per treatment group). 16
36 hours after the last daily dose of TCE, mice received tritiated thymidine and were sacrificed 6
37 hours later. Hepatic DNA was extracted form whole liver and standard histopathology was also
38 performed. Hepatic DNA content and cellular distributions were also determined for thymidine
39 uptake using autoradiography of tissue sections. Tritiated thymidine incorporation into DNA
40 was determined by microscopic observations of autoradiography slides and reported as positive
41 cells per 100 (200X power) fields.
42

1   Changes in the treatment groups were reported to "include an increase in eosinophilic
2   cytoplasmic staining of hepatocytes located near central veins, accompanied by loss of
3   cytoplasmic vacuolization. Intermediate zones appeared normal and no changes were noted in
4   portal triad areas. Male and female mice given 1000 mg/kg body weight TCE exhibited
5   apoptosis located near central veins. No evidence of cellular proliferation was seen in the portal
6   areas. No evidence of increased lipofuscin was seen in liver sections from male and female mice
7   treated with TCE. Evaluation of cell death in male and female mice receiving TCE was
8   performed by enumerating apoptoses." The apoptoses "did not appear to be in proportion to the
9   applied TCE dose given to male or female mice." The mean number of apopotosis per 100 (400
10   X) fields in each group of 4 animals (male mice) was 0, 0, 0, 1 and 8 for control, 100, 250, 500,
11   and 1000 mg/kg TCE treated groups, respectively. Variations in number of apoptoses between
12   mice were not given by the authors. Feulgen stain was <1 for all doses except for 9 at 1000
13   mg/kg.
14
15   Mitotic figure were reported to be "frequently seen in liver sections from both male and
16   female mice treated with TCE. Dividing cells were most often found in the intermediate zone
17   and resembled mature hepatocytes. Incorporation of the radiolabel into cells located near the
18   portal triad areas was rare. In general mitotic figures were very rare, but when found they were
19   usually located in the intermediate zone. Little or no incorporation of label was seen in areas
20   near the bile duct epithelia or in areas close to the portal triad." No quantitative description of
21   mitotic index was reported by the authors but this description is consistent with there being
22   replication of mature hepatocytes induced by TCE.
23
24   The distribution of tritiated thymidine was given for specific cell types in the livers of 5
25   animals per treatment group and radiolabel was reported to be predominantly associated with
26   perisinusoidal cell in control mice. The authors state that the label was more often found in cells
27   resembling mature hepatocytes. The mean number of labeled cells in autoradiographs per 100
28   (200X power) fields was reported to be ~ 125 and ~ 150 labeled perisinusoidal cells in controls
29   male and female mice, respectively. The authors do not give any standard deviations for the
30   female perisinusoidal data except for the 1000 mg/kg exposure group. For mature hepatocytes,
31   the mean baseline level of cell labeling for control male and female mice were reported to be ~
32   65 and ~ 90 labeled cells, respectively. Although the baseline levels of hepatocyte labeling were
33   reported to differ between male and female mice, the mean peak level of labeling was similar at
34   ~ 250 labeled cells for male and female mice treated with TCE. Thus, in male mouse liver, the
35   number of labeled cells increased ~ 2-fold of control levels after 500 and 1000 mg/kg TCE and
36   in female mouse liver increased ~ 4-fold of control levels after 250, 500 and 1000 mg/kg TCE in
37   female mouse liver hepatocytes over their respective control levels.
38
39   Incorporation of tritiated thymidine into DNA extracted from whole liver in male and
40   female mice was reported to be significantly elevated after TCE treatment but, unlike the
41   autoradiographic data, there was no difference between genders and the mean peak level of
42   tritiated thymidine incorporation occurred at 250 mg/kg TCE treatment and remained constant
43   for the 500 and 1000 mg/kg treated groups. Increased thymidine incorporation into DNA

1   extracted from liver of male and female mice were reported to show a very large standard
2   deviation with TCE treatment (e.g., at 100 mg/kg TCE exposure, male mice had a mean of ~130
3   dpm tritiated thymidine/ microgram DNA with the upper bound of the standard deviation to be
4   225 dpm). The increased thymidine incorporation peaked at a level that was a little less than 2-
5   fold of control level. Thus, for both male and female mice both autoradiographs and total
6   hepatic DNA were reported to show that male and female mice had similar peaks of increased
7   thymidine incorporation after TCE exposure that reached a plateau at the 250 mg/kg TCE
8   exposure level and did not increase with increasing exposure concentration. These data also
9   indicate a very small population undergoing mitosis due to TCE exposure after 10 days of
10   exposure. If higher levels of hepatocyte replication had occurred earlier, such levels were not
11   sustained by 10 days of TCE exposure. More importantly, these data suggest that tritiated
12   thymidine levels were targeted to mature hepatocytes and in areas of the liver where greater
13   levels of polyploidization. The ages and weights of the mice were described by these authors,
14   unlike Elcombe et al, and a different strain was used. However, these results are consistent with
15   those of Elcombe in regard to the magnitude of thymidine incorporation induced by TCE
16   treatment and the lack of a dose response once a relative low level of exposure has been
17   exceeded.
18
19   The total liver DNA content of male and female mice treated with TCE were also
20   determined with the total micrograms DNA/g liver reported to be ~ 4 microgram/g for female
21   control mice and ~ 2 micrograms/g for male control mice. Although not statistically significant,
22   the total DNA concentration dropped from ~ 4 to ~ 3 at 100 mg/kg through 1000 mg/kg exposure
23   to TCE in female mice. For male mice the total DNA rose slightly in the 250 and 500 mg/kg
24   groups to ~ 3 micrograms/gram and was similar to control levels at the 100 and 1000 mg/kg TCE
25   treatment groups. The standard deviation in male mice was very large and the number of
26   animals small making quantitative judgments regarding this parameter difficult. The slight
27   decrease reported for female mice would be consistent with the results of Elcombe et al (1985)
28   who describe a slight decrease in hepatic DNA in male mice. However, the reported slight
29   increase in hepatic DNA in male mice in this study is not consistent. Given the small number of
30   animals and the large deviations for female and male mice in the TCE treated groups, this study
31   may not have had the sensitivity to detect slight decreases reported by Elcombe et al.
32
33   In regard to clinical evaluation and weight analyses, both male and female mice given
34   TCE were reported "to appear clinically ill. These mice showed reduced activity and failed to
35   groom. Control mice showed no adverse effects. Female mice were markedly more affected by
36   TCE than their male counterparts. Several deaths of female mice occurred during the course of
37   the TCE treatment regimen." The authors do not give cause of deaths but state that 2 female
38   mice died in the group receiving 250 mg/kg TCE and one in the group receiving 1000 mg/kg
39   during the gavage regimen of the female mice. This appears to be similar gavage error or
40   "accidental death" reported in NTP studies chronic studies of TCE (see below).
41
42   The authors report "no significant difference in the absolute body weight of male and
43   female mice were noted in control groups. Body weight gain in female and males mice treated

1 with TCE was not significantly different from that of control mice. Liver weights in male mice
2 given 500 or 1000 mg/kg and corrected for total body weight were significantly elevated. The
3 corrected liver weights of female mice increase proportionally with the applied dose of TCE."
4 For male mice, liver weights were reported to be 1.40 ± 0.16, 1.38 ± 1.23, 1.48 ± 0.09, $1.61 \pm$
5 0.07, and 1.63 ± 0.11 g for control, 100, 250, 500, and 1000 mg/kg TCE in male mice (n=5),
6 respectively. Body weights were smaller for the 100 mg/kg TCE treatment group although not
7 statistically significant. The liver weights after treatment had a much larger reported standard
8 deviation (1.23 g for 100 mg/kg group vs. <0.16 for all other groups). The % liver/ body weight
9 ratios were reported to be 5.40, 5.41, 5.42, 5.71, and 6.34 % for the same groups in male mice.
10 this represents 1.06-fold and 1.17-fold of control at the 500 and 1000 mg/kg dose. The authors
11 report a statistically significant increase in % liver/body weight ratio only for the 500 mg/kg (i.e.,
12 1.06-fold of control) and 1000 mg/kg (i.e., 1.17-fold of control) TCE exposure groups. The
13 results for female mice liver weights were reported in Table III of the paper, which was
14 mistakenly labeled as for male mice. The reported values for liver weight were 1.03 ± 0.07, 1.05
15 ± 0.10, 1.15 ± 0.98, 1.21 ± 0.18 and 1.34 ± 0.08 g for control, 100, 250, 500, and 1000 mg/kg
16 TCE in female mice (n=5, except for 250 mg/kg and 1000 mg/kg groups), respectively. The %
17 liver/body weight ratios were 5.26, 5.44, 5.68, 6.24, and 6.57% for the same groups. These
18 values represent 1.03-fold, 1.08-fold, 1.19-fold, and 1.25-fold of controls in % liver/body weight.
19 The magnitude of increase in TCE-induced %liver/ body weight ratio in female mice is reflective
20 of the magnitude of the difference in dose up to 1000 mg/kg where it is slightly lower. The
21 female mice were reported to have statistically significant increases in % liver/body ratios at the
22 lowest dose tested (100 mg/kg TCE) after 10 days of TCE exposure that also increased
23 proportionately with dose. Male mice were not reported to have a significant increase in %
24 liver/body weight until 500 mg/kg TCE but a statistically significant increase in liver weight at
25 250 mg/kg TCE. Male mice had a much larger variation in initial body weight than did female
26 mice (range of means of 24.86 to 27.84 g between groups for males or ~11% difference and
27 range of means of 19.48 to 20.27 g for females or ~4%) which may contribute to an apparent
28 lack of effect for a parameter that is dependent on body weight. Only 5 mice were used in each
29 group so the power to detect a change was relatively small.
30
31   The results from this experiment are consistent with those of Elcombe et al (1985) in
32 showing a slight increase in thymidine incorporation (~2-fold of control) and mitotic figures that
33 are rare after TCE exposure. This study also records a lack of apoptosis with TCE treatment
34 except at the highest exposure level (i.e., 1000 mg/kg). The increases in liver weight induced by
35 TCE were reported to be dose-related, especially in female mice where baseline body weights
36 were more consistent. However, the incorporation of tritiated thymidine reached a plateau at 250
37 mg/kg TCE in the DNA of both genders of mice. This study specifically identified where
38 thymidine incorporation and mitotic figures were occurring in TCE-treated livers and noted that
39 the mature hepatocyte that appeared to be primarily affected, as well as in the portion of the liver
40 where mature hepatocytes with higher ploidy are found. The authors note that the "lack of
41 thymidine incorporation in the periportal area, where the liver stem cells are reside," suggesting
42 that the mature hepatocyte is the target of TCE effects on DNA synthesis. This finding is
43 consistent with a change in ploidy accompanying hepatocellular hypertrophy and not just cell

1   proliferation after 10 days of TCE exposure. Like Elcombe et al (1985), these data represent "a
2   snapshot in time" which does not show whether increased cell proliferation may have happened
3   at an earlier time point and then subsided by 10 days. However, like Elcombe et al (1985) it
4   suggests that sustained proliferation is not a feature of TCE exposure and that the level of DNA
5   synthesis (which is very low in quiescent control liver) is increased in a small population of
6   hepatocytes due to TCE exposure that is not dose-dependent (only 2- fold increase over control
7   in animals exposed from 250 to 1000 mg/kg TCE. In regards to toxicity, no evidence of
8   increased lipid peroxidation in TCE-treated animals was reported using histopathologic sections
9   stained to enhance observation of lipofuscin. No necrosis is noted by these authors and the
10 deaths in female mice are likely due to gavage error.
11
12 **2.1.10. Nakajima et al. 2000**
13
14   This study focused on the effect of TCE treatment on PPARα-null mice in terms of
15 peroxisome proliferation but also included information on differences in liver weight between
16 null and wild-type mice, as well as gender-related effects. SV129 wild-type and PPARα-null
17 mice (10 weeks of age) were treated with corn oil or 750 mg/kg TCE in corn oil daily for 2
18 weeks via gavage (n= 6 per group). A small portion of the liver was removed for
19 histopathological examination but the lobe used was not specified by the authors. Liver
20 peroxisome proliferation was reported to be evaluated morphologically using 3,3'-
21 diaminobenzidine (DAB) staining of sections and electron photomicroscopy to detect the volume
22 density of peroxisomes (% of cytoplasm) in 15 micrographs of the pericentral area per liver. A
23 number of β-oxidation enzymes and P450s were analyzed by immunoblot of liver homogenates.
24
25   The final body weights, liver weights and % liver/body weight ratios were reported for all
26 treatment groups. For male mice, vehicle treated PPARα-null mice had slightly lower mean
27 body weights (24.5 ± 1.8 g vs. 25.4 ± 1.9 g [SD]), slightly larger liver weights (1.14 ± 0.13g vs.
28 1.05 ± 0.15g or ~ 9%), and slightly higher % liver/body weight ratios (4.12 ± 0.32 % vs. 4.10 ±
29 0.37%) than wild-type mice. The mean values for final body weights of the groups of mice in
30 this study were reported and were similar which, as demonstrated by the inhalation studies by
31 Kjellstrand et al (1983a) (see Section 2.2.4), is particularly important for determining the effects
32 of TCE treatment on % liver/body weight ratios. For both groups of male mice, 2 weeks of TCE
33 treatment significantly increased both liver weight and % liver/body weight ratios. For male
34 wild type mice the increase in % liver/body weight was 1.50-fold of vehicle control and for male
35 PPARα-null mice the increase was 1.26-fold of control after 2 weeks of TCE treatment. For
36 female mice, vehicle treated PPARα-null mice had slightly higher mean body weights (22.7 ±
37 2.1 g vs. 22.4 ± 2.0 g), slightly larger liver weights (0.98 ± 0.15g vs. 0.95 ± 0.14g or ~ 3%), and
38 slightly higher % liver/body weight ratios (4.32 ± 0.35 % vs. 4.24 ± 0.41%) than wild-type mice.
39 For both groups of female mice, 2 weeks of TCE treatment significantly increased % liver/body
40 weight ratios. For liver weights there was a reporting error for PPARα-null female treated with
41 TCE so that liver weight changes due to TCE treatment cannot be determined for this group. For
42 female wild type mice the increase in % liver/body weight was 1.24-fold of vehicle control and
43 for female PPARα-null mice the increase was 1.26-fold of control after 2 weeks of TCE

1   treatment. Thus for both wild-type and PPARα-null mice, TCE exposure resulted in increased %
2   liver/body weight over controls that was statistically significant after 2 weeks of oral gavage
3   exposure using corn oil as the vehicle. For male mice there was a greater TCE-induced increase
4   in % liver/body weight in wild type than PPARα-null mice (1.50 –fold vs. 1.26-fold of control)
5   that was statistically significant, but for female mice the induction of increased liver weight was
6   statistically increased but the same in wild-type and PPARα-null mice (i.e., both were ~1.25 –
7   fold of control). These date indicate that TCE-induced increases in mouse liver weight were not
8   dependent on a functional PPARα receptor in female mice and suggest that some portion may be
9   in male mice.
10
11   In regard to light and electron microscopic results, the numbers of peroxisomes in
12   hepatocytes of wild-type mice were reported to be increased, especially in the pericentral area of
13   the hepatic lobule, to a similar extent in both males and females (15 micrographs, n= 4 mice).
14   TCE exposure was reported to increase the volume density of peroxisomes 2-fold of control in
15   the pericentral area with no evident change in peroxisomes in the periportal areas, but data was
16   not shown for that area of the liver lobule. In contrast, no increase in peroxisomes was reported
17   to be observed in PPARα-null mice. Therefore, increases in liver weight observed in PPARα-
18   null mice after TCE treatment did not result from peroxisome proliferation. Similarly, the small
19   2–fold increase in peroxisome volume from 2% to 4% of cytoplasmic volume in the pericentral
20   area of the liver lobule in wild-type mice could not have been responsible for the 50% increase
21   liver weight observed in male wild-type mice.
22
23   Although no difference was reported between male and female wildtype mice in regard to
24   TCE-induced peroxisome proliferation in wild type mice, the levels of hepatic enzymes
25   associated with peroxisomes [acyl-CoA (AOX), peroxisomal bifunctional protein (PH),
26   peroxisomal thiolase (PT), very long chain acyl-CoA synthetase (VLACS), and D-type
27   peroxisomal bifunctional protein (DBF), cytosolic enzyme [cytosolic thioesterase II (CTEII)],
28   mitochondrial enzymes [mitochondrial trifunctional protein α subunits α and β(TPα and TPβ)],
29   and microsomal enzymes [Cytochrome P450 4A1 (CYP4A1)] as measured by immunoblot
30   analysis were significantly elevated in male wild type mice (n= 4) by a factor of ~ 2-3, but
31   except for a slight elevation in PH and PT, were reported to not be elevated in female wild-type
32   mice (n= 4). The magnitude of increase in peroxisomal enzymes was similar to that of
33   peroxisomal volume in male mice. No TCE-induced increases in any of these enzymes were
34   reported in male or female PPARα-null mice by the authors. For CYP4A1, an enzyme reported
35   to be induced by peroxisomal proliferators, TCE exposure resulted in a much lower amount in
36   female than male wild-type mice (i.e., 2% of the level induced by TCE in males). However, the
37   expression of catalase was reported to be "nearly constant in all samples" (at most ~ 30%
38   change) which the authors suggested resulted from induction by TCE that was independent of
39   PPARα. The basis for selection of 4 mice for this comparison out of the 6 studied per group was
40   not given by the authors. A comparison of control wild-type and PPARα-null mice showed that
41   in males background levels of the enzymes examined were generally similar except for DBF in
42   which the null mice had values ~ 50% of the wild type controls. A similar decrease was reported
43   for female PPARα-null mice. With regard to gender differences in wild-type mice, females had

1 similar values as males with the exceptions of TPα, TPβ, and CYP2E1 which were in untreated
2 female wild-type mice at a 3.06-fold, 2.38-fold and 1.63-fold for l TPα, TPβ, and CYP2E1 levels
3 over males, respectively. Female PPARα-null mice had increases of 2.50-fold, 1.54-fold, and
4 2.07-fold over male wild-type mice.
5
6   With regard to the induction of TCE metabolizing enzymes (CYP1A2, CYP2E1 and
7 ALDH), CYP1A2 was reported to be decreased by TCE treatment of both male and female wild-
8 type mice but liver CYP2E1 reported to be increased in male mice and constant in female mice
9 which resulted in similar expression level in both genders after TCE treatment. There was no
10 gender difference in ALDH activity reported after TCE exposure and activity was reported to be
11 independent of PPARα. The authors concluded that TCE metabolizing abilities of the liver of
12 male and female mice were similar and therefore poor induction of peroxisomal related enzymes
13 was not due to gender-related differences in TCE metabolism.
14
15   To investigate whether the a gender-related difference peroxisomal enzymes after TCE
16 exposure was due to a lower levels of PPARα and RXRα receptors, western blotting was
17 employed (n= 3).   The level of PPARα protein was reported to be increased in both male wild-
18 type mice with less induction in females (control vs. TCE, 1.00 ± 0.20 vs. 2.17 ± 0.24 in males
19 and 0.95 ± 0.25 vs. 1.44 ± 0.09 in females) after TCE treatment. The hepatic level of RXRα was
20 also reported to be increased in the same manner as PPARα (control vs. TCE, 1.00 ± 0.33 vs.
21 1.92 ± 0.04 in males 0.81 ± 0.16 vs. 1.14 ± 0.10 in females).   Northern blot analysis of hepatic
22 PPARα mRNA was reported to show greater TCE induction in male (2.6-fold of control) than in
23 female (1.5-fold of control) wild-type mice. Thus, males appeared to have higher induction of
24 the two receptor proteins as well as a greater response in peroxisomal enzymes and CYP4A1,
25 even though TCE-induced increases in peroxisomal volume was similar between male and
26 female mice. The increased response in males for induction of the two receptor proteins is
27 consistent with liver weight data that shows some portion of the induction of increased liver
28 weight response in male mice using this paradigm may be due to gender-specific differences in
29 PPARα response. However, as noted below (see Sections 2.2), corn oil vehicle has liver effects
30 alone, especially in the male liver, that have also been associated with PPARα responses.
31
32 **2.1.11. Berman et al. 1995**
33
34   This study included TCE in a suite of compounds used to compare endpoints for
35 toxicological screening methods. Female Fischer 344 rats of 77 days of age (n= 8 per group)
36 were administered TCE in corn oil for 1 day (0, 150, 500, 1500, or 5000 mg/kg/day) or for 14
37 days (0, 50, 150, 500, or 1500 mg/kg/day). Blood samples were taken 24 hours after the last
38 dose and livers were weighed and H&E sections were examined for evidence of parenchymal
39 cell degeneration, necrosis, or hypertrophy. No details were provided by the authors for the
40 extent or severity of the liver affects by histopathological examination. The serum chemistry
41 analysis included LDH, Alkaline phosphatase, ALT, AST, total bilirubin, creatine, and BUN.
42 The starting and ending body weights of the animals or the absolute liver weights were not
43 reported by the authors.

1
2   The results of a multivariate analysis were reported to show a lowest effective dose of
3 1500 mg/kg after 1 day of TCE exposure and 150 mg/kg after 14 days of TCE exposure that was
4 statistically significant. Liver weight and liver weight changes were not reported by the authors
5 but the % liver to body weight ratios were. For the two control groups there was a difference in
6 % liver/body weight of ~ 8% (3.43 ± 0.74 % for the 1-day control group and 3.16 ± 0.41 % for
7 the 14-day control group, mean ± SEM). For the 1-day groups only the 5000 mg/kg group was
8 reported to show a statistically significant difference in % liver/body weight between control and
9 TCE treatment (i.e., ~ 1.08 fold increase). Hepatocellular necrosis was noted to occur in the
10 1500 and 5000 mg/kg groups in 6/7 and 6/8 female rats, respectively but not to occur in lower
11 doses. The extent of necrosis was not noted by the authors for the two groups exhibiting a
12 response after 1 day of exposure. Serum enzymes indicative of liver necrosis were not presented
13 and because only positive results were presented in the paper, presumed to be negative.
14 Therefore, the extent of necrosis was not of a magnitude to affect serum enzyme markers of
15 cellular leakage.
16
17   After 14-days of TCE exposure, there was a dose-related increase reported for %
18 liver/body weight ratios that was statistically significant at all TCE dose levels although the
19 multivariate analysis indicated the lowest effective dose to be 150 mg/kg. The % liver/body
20 weight ratio was 3.16 ± 0.41%, 3.38 ± 0.56%, 3.49 ± 0.69%, 3.82 ± 0.76%, and 4.47 ± 0.66 %
21 for control, 50 mg/kg, 150 mg/kg, 500 mg/kg and 1500 mg/kg TCE exposure levels, respectively
22 after 14 days of exposure. No hepatocellular necrosis was reported at any dose and
23 hepatocellular hypertrophy was reported only at the 1500 mg/kg dose and in all rats. These rat
24 liver weights are 1.07-fold, 1.10-fold, 1.21-fold, and 1.41-fold of controls for the 50, 150, 500
25 and 1500 mg/kg TCE dose groups, respectively. The 7% increase in liver weight at the 50
26 mg/kg dose is approximately the same difference between the two control groups for days 1 and
27 14 treatments. Without the data for starting and final body weights and an examination of
28 whether the control animals had similar body weight, it is impossible to discern whether the
29 reported effects at the low dose of TCE was also reflected differences between the control
30 groups. No serum enzyme levels changes were reported after 14 days of exposure to TCE for
31 any group.
32
33   The authors not that their study provided evidence of liver effects at lower levels than
34 other studies citing Elcombe et al (1985) and Goldsworthy and Popp (1987). They suggest that
35 the differences in sensitivity to TCE between their results and those of these two studies may
36 reflect differences in strain or gender of the rats examined. However, they did not study male rats
37 of this strain concurrently so that differences in gender may have reflected differences between
38 experiments. The increase in liver weight without reporting increases in hepatocellular
39 hypertrophy as well as the lack of necrosis as low doses is consistent with the results of Melnick
40 et al (1987) in male Fischer rats given TCE orally (see Section 2.1.11 below).

1   **2.1.12. Melnick et al. 1987**

2

3   The focus of this study was to assess microencapsulation as a way to expose rodents to
4   substances such as TCE that have issues related to volatilization in drinking water or apparent
5   gavage-related deaths. In this study, liver weight changes, extent of focalized necrosis, and
6   indicators of peroxisome proliferation were reported as metrics of TCE toxicity. TCE (99+ %)
7   was encapsulated in gelatin-sorbitol microcapsuls and was 44.1% TCE w/w. The TCE
8   microcapsules were administered to male Fischer 344 rats (6-week old and weighing between 89
9   and 92 g or ~3% difference) in the diet (0, 0.55, 1.10, 2.21 and 4.42% TCE in the diet) for 14
10 days. The number of animals in each group was 10. A parallel group of animals was
11 administered TCE in corn oil gavage for 14 consecutive days (corn oil control, 0.6, 1.2, and 2.8
12 g/kg/day TCE). The dosage levels of TCE in the gavage study were reported to be "adjusted 5
13 times during the 14-day" treatment period to be similar to the dosage levels of TCE in the feed
14 study. The time-weighted average dosage levels of TCE in the feed study were reported to be
15 0.6, 1.3, 2.2, and 4.8 g/kg/day.

16

17   There was less food consumption reported in the 2.2 and 4.8 g/kg/day dose feed groups
18 which the authors attribute to either palatability or toxicity. There were no deaths in any of the
19 groups treated with microencapsulated TCE while, similar to many other gavage studies of TCE
20 reported in the literature, there were 4 deaths in the high-dose gavage group. Mean body weight
21 gains of the two highest dose groups of the feed study and of the highest dose group of the
22 gavage study were reported to be significantly lower than the mean body weight gains of the
23 respective control groups (i.e., ~ 22 % and ~ 35% reduction at 2.2 and 4.8 g/kg/day in the feed
24 study, respectively, and ~ 33% reduction at 2.8 g/kg/day TCE in the gavage study). After 14
25 days of treatment, liver weights were reported to be 8.1 ± 0.8, 8.4 ± 0.8, 9.5 ± 0.5, 10.1 ± 1.2,
26 8.9 ± 1.3, and 7.4 ± 0.5 g for untreated control, placebo control, 0.6, 1.3, 2.2, and 4.8 g/kg TCE
27 exposed feed groups, respectively. The corresponding % liver/body weight ratios were reported
28 to be 5.2 ± 0.3, 5.3 ± 0.2, 6.0 ± 0.3, 6.5 ± 0.5, 7.0 ± 0.9, and 7.1 ± 0.5 % for untreated control,
29 placebo control, 0.6, 1.3, 2.2, and 4.8 g/kg TCE exposed groups, respectively.   The increased %
30 liver/body weight ratio represents 1.13-fold, 1.23-fold, 1.32-fold, and 1.34-fold of placebo
31 controls, respectively. For the gavage experiment, after 14 days of treatment liver weights were
32 reported to be 7.1 ± 1.3, 9.3 ± 1.2, 9.1 ± 0.9, and 7.7 ± 0.4 g for corn oil control, 0.6, 1.2, and 2.8
33 g/kg TCE exposed groups, respectively. The corresponding % liver/body weight ratios were
34 reported to be 5.0 ± 0.4, 6.0 ± 0.4, 6.1 ± 0.3, and 7.3 ± 0.5 % for corn oil control, 0.6, 1.2, and
35 2.8 g/kg TCE exposed groups, respectively. The % liver/body weight ratios represent 1.20-fold,
36 1.22-fold, and 1.46-fold of corn oil controls, respectively. The 2.8 g/kg TCE gavage results are
37 reflective of the 6 surviving animals in the group rather than 10 animals in the rest of the groups.
38 There was no explanation given by the authors for the lower liver weights in the control gavage
39 group than the placebo control in the feed group (i.e., 20% difference) although the initial and
40 final body weights appeared to be similar. The decreased body weights in the feed and gavage
41 study are reflective if TCE systemic toxicity and appeared to affect the TCE-induced liver weight
42 increases in those groups.

1   The authors reported that the only treatment-related lesion observed microscopically in
2   rats from either dosed-feed or gavage groups was individual cell necrosis of the liver with the
3   frequency and severity of this lesion similar at each dosage levels of TCE administered
4   microencapsulated in the feed or in corn oil. Using a scale of minimal = 1-3 necrotic
5   hepatocytes/10 microscopic 200X fields, mild = 4 – 7 necrotic necrotic hepatocytes/10
6   microscopic 200X fields, and moderate = 8-12 necrotic hepatocytes/10 microscopic 200X fields,
7   the frequency of lesion was 0-1/10 for controls, 2/10 for 0.6 and 1.3 g/kg and 9/10 for 2.2 and 4.8
8   g/kg feed groups. The mean severity was reported to be 0.0-0.1 for controls, 0.3-0.4 for 0.6 and
9   1.3 g/kg, and 2.0 -2.5 for 2.2 and 4.8 g/kg feed groups. For the corn oil gavage study, the corn
10   oil control and 0.6 g/kg groups were reported to have a frequency of 0 lesions/10 animals, the
11   1.2 g/kg group a frequency of 1/10 animals, while the 2.8 g/kg group to have a frequency of 5/6
12   animals. The mean severity score was reported to be 0 for the control and 0.6 g/kg groups, 0.1
13   for the 1.2 g/kg groups, and 1.8 for the remaining 6 animals in the 2.8 g/kg group. The
14   individual cell necrosis was reported to be randomly distributed throughout the liver lobule with
15   the change to not be accompanied by an inflammatory response. The authors also report that
16   there was no histologic evidence of cellular hypertrophy or edema in hepatic parenchymal cells.
17   Thus, although there appeared to be TCE-treatment related increases in focal necrosis after 14
18   days of exposure, the extent was even at the highest doses mild and involved few hepatocytes.
19
20   Microsomal NADPH cytochrome c-reductase was reported to be elevated in the 2.2 and
21   4.8 g/kg feed groups and in the 1.2 and 2.8 g/kg gavage groups. Cytochrome P450 levels were
22   reported to be elevated only in the two highest dose groups of the feed study. The authors
23   reported a dose-related increase in peroxisome PCO and catalase activities in liver homogenates
24   from rats treated with TCE microcapsules or by gavage and that treatment with corn oil alone,
25   but not placebo capsules, caused a slight increase in PCO activity. After 14 days of treatment,
26   PCO activities were reported to be 270 ± 12, 242 ± 17, 298 ± 64, 424 ± 55, 651 ± 148, and $999 \pm$
27   266 nmole H_2O_2 produced/ min/g liver for untreated control, placebo control, 0.6, 1.3, 2.2, and
28   4.8 g/kg TCE exposed feed groups, respectively. This represents 1.23-fold, 1.75-fold, 2.69-fold,
29   and 4.13-fold of placebo controls, respectively. After 14 days of treatment, catalase activities
30   were reported to be 8.49 ± 0.81, 7.98 ± 1.62, 8.49 ± 1.92, 8.59 ± 1.31, 13.03 ± 2.01, and $15.76 \pm$
31   1.11 nmole H_2O_2 produced/ min/g liver for untreated control, placebo control, 0.6, 1.3, 2.2, and
32   4.8 g/kg TCE exposed groups, respectively. This represents 1.06-fold, 1.07-fold, 1.63-fold, and
33   1.97-fold of placebo controls, respectively. Thus, although reported to be dose related, only the
34   two highest exposure levels of TCE increased catalase activity and to a smaller extent than PCO
35   activity in microencapsulated TCE fed rats. For the gavage experiment, after 14 days of
36   treatment PCO activities were reported to be 318 ± 27, 369 ± 26, 413 ± 40, and 1002 ± 271
37   nmole H_2O_2 produced/ min/g liver for corn oil control, 0.6, 1.2, and 2.8 g/kg TCE exposed
38   groups, respectively. This represents 1.16-fold, 1.29-fold, and 3.15-fold of corn oil controls.
39   After 14 days of treatment, catalase activities were reported to be 8.59 ± 0.91, 10.10 ± 1.82,
40   12.83 ± 3.43, and 13.54 ± 2.32 nmole H_2O_2 produced/ min/g liver for corn oil control, 0.6, 1.2,
41   and 2.8 g/kg TCE exposed groups, respectively. This represents 1.18-fold, 1.49-fold, and 1.58-
42   fold of corn oil controls. As stated by the authors the corn oil vehicle appeared to elevate
43   catalase activities and PCO activities.

1   In regard to dose-response, liver and body weight were affected by decreased body
2 weight gain in the higher dosed animals in this experiment (i.e., 2.2 g/kg/day TCE exposure and
3 above) and by gavage related deaths in the highest-dosed group. The lower liver weight in the
4 gavage control group also may have affected the determination of the magnitude of TCE-related
5 liver weight gain at that dose. At the 2 doses, below which body weight gain was affected, there
6 appeared to be an approximately 20% increase in % liver/body weight ratio in the gavage study
7 and a 13% and 23 % weight increase in the feed study.   The extent of PCO activity appeared to
8 increase more steeply with dose in the feed study than did liver weight gain (i.e., a 1.23-fold of
9 liver/body weight ratio at 1.3 g/kg/day corresponded with a 1.75-fold PCO activity over control).
10 At the two highest doses in the feed study the increase in PCO activity was 2.69-fold and 4.13-
11 fold of control but the increase in liver weight was not more than 34%. For the gavage study
12 there was also a steeper increase in PCO activity than liver weight gain. For catalase activity, the
13 increase was slightly less than that of liver/body weight ratio % for the two doses that did not
14 decrease body weight gain in the feed study. In the gavage study they were about the same. In
15 regard to what the cause of liver weight gain was, the authors report that there was no histologic
16 evidence of cellular hypertrophy or edema in hepatic parenchymal cells and do not describe
17 indicators of hepatocellular proliferation or increased polyploidy. Accordingly, the cause of liver
18 weight gain after TCE exposure in this paradigm is not readily apparent.
19
20 **2.1.13. Laughter et al. 2004**
21
22   Although the focus of the study was an exploration of potential MOAs for TCE effects
23 through macroarray transcript profiling (see Section 3.1.2. for discussions of limitations of this
24 approach and especially the need for phenotypic anchoring, Section 3.4.1.3 for use of PPARα
25 knockout mice, and Section 3.4.2.2 for discussion of genetic profiling data for TCE), information
26 was reported regarding changes in the liver weight of PPARα-null mouse and their background
27 strains. SV129 wild type and PPARα-null male mice (9 ± 1.5 weeks of age) were treated with 3
28 daily doses of TCE in 0.1% methyl cellulose for either 3 days or 3 weeks (n= 4-5/group). Thus,
29 this paradigm does not use corn oil, which has been noted to affect toxicity (see Section 2.2
30 below), but is not comparable to other paradigms that administer the total dose in one daily
31 gavage administration rather than to give the same cumulative dose but in 3 daily doses of lower
32 concentration. The initial or final body weights of the mice were not reported. Thus, the effects
33 of systemic toxicity from TCE exposure on body weight and the influence of differences in
34 initial body weight on % liver/body weight determinations cannot be made. For the 3-day study,
35 mice were administered 1500 mg/kg TCE or vehicle control. For the 3-week study mice were
36 administered 0, 10, 50, 125, 500, 1000, or 1500 mg/kg TCE 5 days a week except for 4 day/
37 week on the last week of the experiment. In a separate study mice were given TCA or DCA at
38 0.25, 0.5, 1, or 2 g/l (pH ~ 7) in the drinking water for 7 days. For each animal a block of the
39 left, anterior right, and median liver lobes was reported to be fixed in formalin with 5 sections
40 stained for H&E and examined by light microscopy. The remaining liver samples were
41 combined and used as homogenates for transcript arrays. In the 3-week study, BrdU was
42 administered via miniosmotic pump on day one of week 3 and sections of the liver assessed for
43 BrdU incorporation in at least 1000 cells per animal in 10 – 15 fields.

1   Although initial body weights, final body weights, and the liver weights were not
2 reported, the % liver/ body ratios were. In the 3-day study, control wild type and PPARα-null
3 mice were reported to have similar % liver/body weight ratios of ~ 4.5%. These animals were ~
4 10 weeks of age upon sacrifice. However at the end of the 3-week experiment the % liver/body
5 weight ratios were increased in the PPARα-null male mice and were 5.1%. There was also a
6 slight difference in the % liver/body weight ratios in the 1-week study (4.3 ± 0.4 % vs. 4.6 ± 0.2
7 % for wild-type and PPARα-null mice, respectively). These results are consistent with an
8 increasing baseline of hepatic steatosis with age in the PPARα-null mice and increase in liver
9 weight. In the 3-day study, the mean reported the % liver/body ratio was 1.4-fold of the animals
10 tested with TCE in comparison to the control level.   In the PPARα-null mice there was a 1.07-
11 fold of control level reported by the authors to not be statistically significant. However, given
12 the low number of animals tested (the authors give only that 4-5 animals were tested per group
13 without identification as to which groups has 4 animals and which had 5), the ability of this study
14 to discern a statistically significant difference is limited. In the 3-week study, wild-type mice
15 exposed to various concentrations of TCE had % liver/body weights that were within ~ 2% of
16 control values except for the 1000 mg/kg and 1500 mg/kg groups that were ~ 1.18 – fold and
17 1.30 - fold of control levels, respectively. For the PPARα-null mice exposed to TCE for 3
18 weeks, the variability in % liver/body weight was greater than that of the wild-type mice in most
19 of the groups. The baseline level % liver/body weight was 1.16-fold in the PPARα-null mice in
20 comparison to wild-type mice. At the 1500 mg/kg TCE exposure level % liver/body weights
21 were not recorded because of the death of the null mice at this level. The authors reported that at
22 the 1500 mg/kg level all PPARα-null mice were moribund and had to be removed from the
23 study. However, at 1000 mg/kg TCE exposure level there was a 1.10-fold of control %
24 liver/body weight value that was reported to not be statistically significant. However, as noted
25 above, the power of the study was limited due to low numbers of animals and increased
26 variability in the null mice groups. The % liver/body weight reported in this study was actually
27 greater in the null mice than the wild-type male mice at the 1000 mg/kg TCE exposure level (5.6
28 ± 0.4 % vs. 5.2 ± 0.5%, for null and wild-type mice, respectively).   Thus, at 1-week and at 3-
29 weeks, TCE appeared to induce increases in liver weight in PPARα-null mice, although not
30 reaching statistical significance in this study, with concurrent background of increased liver
31 weight reported in the knockout mice.   At 1000 mg/kg TCE exposure for 3 weeks, % liver/body
32 weight was reported to be 1.18-fold in wild-type and 1.10-fold in null mice of control values. As
33 discussed above, Nakajima et al. (2000) reported statistically significant increased liver weight in
34 both wild-type and PPARα-null mice after 2 weeks of exposure with less TCE-induced liver
35 weight increases in the knockout mice (see Section 2.1.10). They also used more mice, carefully
36 matched to weights of their mice, and used a single dose of TCE each day with corn oil gavage.
37
38   The authors noted that inspection of the livers and kidneys of the moribund null mice,
39 who were removed from the 3-week study, "did not reveal any overt signs of toxicity in this dose
40 group that would lead to morbidity" but did not show the data and did not indicate when the
41 animals were affected and removed. For the wild-type mice exposed to the same concentration
42 (1500 mg/kg) but whose survival was not affected by TCE exposure, the authors reported that at
43 the 1500 mg/kg dose these mice exhibited mild granuloma formation with calcification or mild

1 hepatocyte degeneration but gave not other details or quantitative information as to the extent of
2 the lesions or what parts of the liver lobule were affected. The authors noted that "wild-type
3 mice administered 1000 and 1500 mg/kg exhibited centrilobular hypertrophy" and that "the mice
4 in the other groups did not exhibit any gross pathological changes after TCE exposure." Thus,
5 the hepatocellular hypertrophy reported in this study for TCE appeared to be correlated with
6 increases in % liver/body weight in wild-type mice. In regard to the PPARα-null mice, the
7 authors stated that "differences in the liver to body weights in the control PPARα-null mice
8 between Study 1 and 2 [sic the 3-day and 3-week studies] were noted and may be due to
9 differences in the degree of steatosis that commonly occurs in this strain." Further mention of
10 the background pathology due to knockout of the PPARα was not discussed. The increased %
11 liver/body weight reported between control and 1000 mg/kg TCE exposed mice (5.1% vs. 5.6%)
12 was not accompanied by any discussion of pathological changes that could have accounted for
13 the change.
14
15   Direct comparisons of the effects of TCE, DCA, and TCA cannot be made from this
16 study as they were not studied for similar durations of exposure. However, while TCE induced
17 increased in % liver/body weight ratios after 3 days and 3 weeks of exposure in wild-type mice
18 at the highest dose levels, for TCA exposure % liver/body weight after 1 week exposure in
19 drinking water was slightly elevated at all dose levels with no dose-response (~10% increase),
20 and for DCA exposure in drinking water a similar elevation in % liver/body weight was also
21 reported for the 0.25, 0.5, and 1.0 g/l dose levels (~ 11%) and that was increased at the 2.0 g/l
22 level by ~25% reaching statistical significance.   The authors interpret this data to show no TCA-
23 related changes in wild-type mice but the limited power of the study makes quantitative
24 conclusions difficult. For PPARα-null mice all there was a slight decrease in % liver/body
25 weight between control and TCA treated mice at the doses tested (~2%). For DCA treated mice,
26 all treatment levels of DCA were reported to induce a higher % liver/body weight ratio of at least
27 ~ 5% with a 13% increase at the 2.0 g/l level. Again the limited power of the study and the lack
28 of data for TCE at similar durations of exposure as those studied for TCA and DCA makes
29 quantitative conclusions difficult and comparisons between the chemicals difficult. However,
30 the pattern of increased % liver/body weight appears to be more similar between TCE and DCA
31 than TCA in both wild-type and PPARα-null mice. In terms of histological description of
32 effects, the authors note that "livers from the 2 g/l DCA-treated wild-type and PPARα-null mice
33 had hepatocyte cytoplasmic rarefication probably due to an increase in glycogen accumulation."
34 However, no special procedures are staining were performed to validate the assumption in this
35 experiment. No other pathological descriptions of the DCA treatment groups were provided. In
36 regard to TCA, the authors noted that "the livers from wild-type but not PPARα-null mice
37 exposed to 2.0g/l TCA exhibited centrilobular hepatocyte hypertrophy." No quantitative
38 estimate of this effect was given and although the extent of increase of % liver/body weight was
39 similar for all dose levels of TCA, there is no indication from the study that lower concentrations
40 of TCA also increased hepatocellular hypertrophy or why there was no concurrent increase in
41 liver weight at the highest dose of TCA in which hepatocellular hypertrophy was reported.
42 Thus, reports of hepatocellular hypertrophy for DCA and TCA in the 1-week study were not
43 correlated with changes in % liver/body weight.

1   For control animals, BrdU incorporation in the last week of the 3-week study was
2   reported to be at a higher baseline level in PPARα-null mice than wild-type mice (~ 2.5 –fold).
3   For wild type mice the authors reported a statistically significant increase at 500 and 1000 mg/kg
4   TCE at levels of ~1% and ~ 4.5% hepatocytes incorporating the label after 5 days of BrdU
5   incorporation. Whether this measure of DNA synthesis is representative of cellular proliferation
6   or of polyploidization was not examined by the authors. Even at 1000 mg/kg TCE the % of cells
7   that had incorporated BrdU was less than 5% of hepatocytes in wild type mice. The magnitude
8   % liver/body weight ratio change at this exposure level was 4-fold greater than that of
9   hepatocytes undergoing DNA synthesis (16% increase in % liver/body weight ratio vs. 4%
10   increase in DNA synthesis). The ~ 1% of hepatocytes undergoing DNA synthesis at the 500
11   mg/kg TCE level, reported to be statistically significant by the authors, was not correlated with a
12   concurrent increase in % liver/body weight ratio. Thus, TCE-induced changes in liver weight
13   were not correlated with increases in DNA synthesis in wild-type mice after 3 weeks of TCE
14   exposure. For PPARα-null mice, there was a ~ 3-fold of control value for the % of hepatocytes
15   undergoing DNA synthesis at the 1000 mg/kg TCE exposure level. The higher baseline level in
16   the null mouse, large variability in response at this exposure level, and low power of this
17   experimental design limited the ability to detect statistical significance of this effect although the
18   level was greater than that reported for the 500 mg/kg TCE exposure in wild-type mice that was
19   statistically significant. Thus, TCE appeared to induce an increase in DNA synthesis in PPARα-
20   null mice, albeit at a lower level than wild-type mice. However, the ~ 2% increase in % of
21   hepatocytes undergoing DNA synthesis during the 3rd week of a 3-week exposure to 1000 mg/kg
22   TCE in PPARα-null mice was insufficient to account for the ~10% observed increase in liver
23   weight. For wild-type and PPARα-null mice, the magnitude of TCE-induced increases in liver
24   weight were 4-5 fold higher than that of increases in DNA-synthesis under this paradigm and in
25   both types of mice, a relatively small portion of hepatocytes were undergoing DNA synthesis
26   during the last week of a 3-week exposure duration. Whether the increases in liver weight could
27   have resulted from and early burst of DNA synthesis as well as whether the DNA synthesis
28   results reported here represents either proliferation or polyploidization, cannot be determined
29   from this experiment. Because of the differences in exposure protocol (i.e. use of 3 daily doses
30   in methylcellulose rather than one dose in corn oil) the time course of the transient increase in
31   DNA synthesis reported cannot be assumed to be the same for this experiment and others.
32
33   Not only were PPARα-null mice different than wild-type mice in terms of background
34   levels of liver weights, and hepatic steatosis, but this study reported that background levels of
35   PCO activity to be highly variable and in some instances different between wild-type and null
36   mice. There was reported to be ~ 6-fold PCO activity in PPARα-null control mice in comparison
37   to wild-type control mice in the 1-week DCA/TCA experiment (~ 0.15 vs. 0.85 units of
38   activity/g protein). However, in the same figure a second set of data is reported for control mice
39   for comparison to WY-14,643 treatment in which PCO activity was slightly decreased in
40   PPARα-null control mice vs. wild-type controls (~ 0.40 vs. 0.65 units of activity/g protein). In
41   the experimental design description of the paper, WY-14,643 treatment and a separate control
42   were not described as part of the 1 –week DCA/TCA experiment. For the only experiment in
43   which PCO activity was compared between wild-type and PPARα-null mice exposed to TCE

1　(i.e., 3-day exposure study), there was a reported increased over the control value of ~ 2.5 fold
2　that was reported to be statistically significant at 1500 mg/kg TCE (1.5 vs. 0.60 units of
3　activity/g protein). For control mice in the 3-day TCE experiment there was an increase in this
4　activity in PPARα-null mice in comparison to wild-type mice (~ 0.60 vs. 0.35 units of activity/g
5　protein).   While not statistically significant, there appeared to be a slight increase in PCO
6　activity after 1500 mg/kg TCE exposure for 3 days in PPARα-null mice of ~ 30%.   However, as
7　noted above the background levels of this enzyme activity varied widely between the
8　experiments with not only values for control animals varying as much as 6-fold (i.e. for PPARα-
9　null mice) but also for WY-14,643 administration. There was a 6.6-fold difference in PCO
10　results for WY-14,643 in PPARα-null mice at the same concentration of WY-14,643 in the 3-day
11　and 1 week experiment, and a 1.44-fold difference in results in wild-type mice in these two data
12　sets.
13
14　**2.1.14. Ramdhan et al. 2008**
15
16　　Ramdhan et al (2008) examined the role of CYP 2E1 in TCE-induced hepatotoxicity,
17　using cyp2E1+/+ (wild-type) and cyp2E1-/- (null) Sv/129 male mice (6/group) which were
18　exposed for 7 days to 0, 1000, or 2000 ppm TCE by inhalation for 8 hours/day (Ramdhan et al.
19　2008). The exposure concentrations are noted by the authors to be much higher than
20　occupational exposures and to have increased liver toxicity after 8 hours of exposure as
21　measured by plasma AST levels. To put this exposure concentration into perspective, the
22　Kjellstrand et al (1983a; 1983 b) inhalation studies for 30 days showed that these levels were
23　well above the 150 ppm exposure levels in male mice that induced systemic toxicity. Nunes also
24　reported hepatic necrosis up to 4% in rats at 2000 ppm for just 8 hours not 7 days. AST and
25　ALT were measured at sacrifice. Histological changes were scored using a qualitative scale of 0
26　= no necrosis, 1 = minimal as defined as only occasional necrotic cells in any lobule, 2 = mild as
27　defined as less than one-third of the lobule structure affected, 3= moderate as defined as between
28　one-third and two-thirds of the lobule structure affected and 4 = severe defined as greater than
29　two-thirds of the lobule structure affected. Real-time PCR was reported for mRNA encoding a
30　number of receptors and proteins. Total RNA and Western Blot analysis was obtained from
31　whole liver homogenates The changes in mRNA expression were reported as means for 6 mice
32　per group after normalization to a level of β-actin mRNA expression and were shown relative to
33　the control level in the CYP2E1 wild type mice.
34
35　　The deletion of the CYP2E1 gene in the null mouse had profound effects on liver weight.
36　The body were was significantly increased in control CYP2E1 -/- mice in comparison to wild
37　type controls (24.48 g ± 1.44 g for null mice vs. 23.66 ± 2.44 g , m ± SD). This represents a
38　3.5% increase over wild type mice. However, the liver weight was reported in the CYP2E1 -/-
39　mice to be 1.32-fold of that of CYP2E1 +/+ mice (1.45 ± 0.10 g vs. 1.10 ± 0.14g). The %
40　liver/body weight ratio was 5.47% vs. 4.63 % or 1.18-fold of wild type control for the null mice.
41　The authors report that 1000 ppm and 2000 ppm TCE treatment did induce a statistically
42　significant change body weight for null or wild type mice. However there was an increase in
43　body weight in the wild type mice (i.e., 23.66 ± 2.44, 24.52 ± 1.17, and 24.99 ± 1.78 for control,

1   1000 ppm, and 2000 ppm groups, respectively) and an increase in the variability in response in
2   the null mice (i.e., 24.48 ± 1.44, 24.55 ± 2.26, and 24.99 ± 4.05, for control, 1000 ppm, and 2000
3   ppm exposure groups, respectively). The % liver/body weight was 5.47 ± 0.23, 5.51 ± 0.27, and
4   5.58 ± 0.70 % for control, 1000 ppm and 2000 ppm the CYP2E -/- mice, respectively. The %
5   liver/body weight was 4.63 ± 0.13, 6.62 ± 0.40, and 7.24 ± 0.84 % for control, 1000 ppm, and
6   2000 ppm wild type mice, respectively. Therefore, while there appeared to be little difference in
7   the TCE and control exposures for % liver/body weights in the CYP2E1 -/- mice (2%) there was
8   a 1.56-fold of control level after 2000 ppm in the wild type mice after 7 days of inhalation
9   exposure.
10
11   The authors reported that "in general, the urinary TCE level in CYP2E1 -/- mice was less
12   than half that in CYP2E1 +/+ mice: urinary TCA levels in the former were about one-fourth
13   those in the latter." Of note is the large variability in urinary TCE detected in the 2000 ppm TCE
14   exposed wild type mice , especially after day 4, and that in general the amount of TCE in the
15   urine appeared to be greatest after the 1st day of exposure and steadily declined between 1 and 7
16   days (i.e., ~ 45% decline at 2000 ppm and a ~70% decline at 1000 ppm) in the wild type mice.
17   The amount of TCE in the urine was proportional to the difference in dose at days 1 and 5 (i.e., a
18   2-fold difference in dose resulted in a 2-fold difference in TCE detected in the urine). As the
19   detection of TCE in the urine declined with time, the amount of TCA was reported to steadily
20   increase between days 1 and 7 (e.g., from ~ 3 mg TCA after the 1st day to ~ 5.5 mg after 7 days
21   after 2000 ppm exposure in wild-type mice). However, unlike TCE, there was a with much
22   smaller differences in response between the two TCE exposure levels (i.e., a 12 – 44% or 1.12-
23   fold to 1.44-fold difference in TCA levels in the urine at days 1-7 for exposure concentrations
24   that differ by a factor of 2). This could be indicative of saturation in metabolism and TCA
25   clearance into urine at these high concentrations levels.   The authors note that their results
26   suggest that the metabolism of TCE in both null and wild-type mice may have reached saturation
27   at 1000 ppm TCE.
28
29   For ALT and AST activities in CYP2E1 -/- or CYP2E1 +/+ mice, both liver enzymes
30   were significantly elevated only at the 2000 ppm level in CYP2E1 +/+ mice. Although the
31   increases in excreted TCA in the urine differed by only ~ 33% between the 1000 and 2000 ppm
32   levels, liver enzyme levels in plasma differed by a much greater extent after 7 days exposure
33   between the 1000 and 2000 ppm groups of CYP2E1 +/+ mice (i.e., 1.26-fold and 1.83-fold of
34   control (ALT) and 1.40-fold and 2.20-fold of control (AST) for 1000 ppm and 2000 ppm TCE
35   exposure levels, respectively). The authors reported a correlation between plasma ALT and both
36   TCE (r= 0.7331) and TCA (r= 0.8169) levels but do not report details of what data were included
37   in the correlation (i.e., were data from CYP2E1 +/+ mice combined with those of the CYP2E1 -/-
38   mice and were control values included with treated values?).
39
40   The authors show photomicrograph of a section of liver from control CYP2E1 +/+ and
41   CYP2E1 -/- mice and describe the histological structure of the liver to appear normal. This
42   raises the question as to the cause of the hepatomegaly for the CYP2E1 mice in which the liver
43   weight was increased by a third. The qualitative scoring for each of the 6 animals per group

1   showed that none of the CYP2E1 -/- control or treated mice showed evidence of necrosis. For
2   the CYP2E1 +/+ mice there was no necrosis reported in the control mice and in 3/6 mice treated
3   with 1000 ppm TCE. Of the 3 mice that were reported to have necrosis, the score was reported
4   as 1-2 for 2 mice and 1 for the third. It is not clear what a score of 1-2 represented given the
5   criteria for each score given by the authors which defined a score of 1 as minimal and one of 2 as
6   mild. For the 2000 ppm TCE-exposed mice, all mice were reported to have at least minimal
7   necrosis (i.e., 4 mice were reported to have scores of 1-2, one mouse a score of 3 and one mouse
8   a score of 1). What is clear from the histopathology data is that there appeared to be great
9   heterogeneity of response between the 6 animals in each TCE-exposure group in CYP2E1 +/+
10 mice and that there was a greater necrotic response in the 2000 ppm exposed mice than the 1000
11 ppm mice. These results are consistent with the liver enzyme data but not consistent with the
12 small difference between the 1000 ppm and 2000 ppm exposure groups for TCA content in urine
13 and by analogy, metabolism of TCE to TCA. A strength of this study is that it reports the
14 histological data for each animal so that the heterogeneity of liver response can be observed (e.g.,
15 the extent of liver necrosis was reported to range from only occasional necrotic cells in any
16 lobule to between one-third and two-thirds of the lobular structure affected after 2000 ppm TCE
17 exposure for 7 days). Immunohistochemical analysis was reported to show that CYP2E1 was
18 expressed mainly around the centrilobular area in CYP2E1 +/+ mice where necrotic changes
19 were observed after TCE treatment.
20
21   Given the large variability in response within the liver after TCE exposure in CYP2E1
22 mice, phenotypic anchoring becomes especially important for the interpretation of mRNA
23 expression studies (see Sections 1.1 and 3.1.2. for macroarray transcript profiling limitations and
24 the need for phenotypic anchoring). However, the data for mRNA expression of PPARα,
25 peroxisomal bifunctional protein (hydratase+3-hydroxyacyl-CoA dehydrogenase),very long
26 chain acyl-CoA dehydrogenase (VLCAD), CYP4A10, NFκB (p65, P50, P52), and IκBα was
27 reported at the means ± SD for 6 mice per group and represented total liver homogenates. A
28 strength of the study was that they did not pool their RNA and can show means and standard
29 deviations between treatment groups. The low numbers of animals tested however limits the
30 ability to detect statistically significance of the response. By reporting the means, differences in
31 the responses within dose groups was limited and reflected differential response and involvement
32 for different portions of the liver lobule and for the responses of the heterogeneous group of
33 liver cells populating the liver. The authors reported that they normalized values to the level of
34 β-actin mRNA in same preparation with a value of 1 assigned as the mean from each control
35 group. The values for mRNA and protein expression reported in the figures appeared to have all
36 been normalized to the control values for the CYP2E1-/- mice. Although all of the CYP2E1 -/-
37 control values were reported as a value of 1, the control values for the CYP2E1+/+ mice differed
38 with the greatest difference being presented for the CYP4A10-mRNA (i.e., the control level of
39 CYP4A10 mRNA was ~ 3-fold higher in the CYP2E1+/+ mice than the CYP2E1 -/- mice).
40 Further characterization of the CYP2E1 mouse model was not provided by the authors.
41
42   The mean expression of PPARα mRNA was reported slightly reduced after TCE
43 treatment in CYP2E1-/- mice (i.e., 0.72-fold and 0.78-fold of control after 1000 and 2000 ppm

1   TCE exposure, respectively). The CYP2E1 -/- mice had a higher baseline of PPARα mRNA
2   expression than the CYP2E1+/+ mice (i.e., the control level of the CYP2E1 -/- mice was 1.5-fold
3   of the CYP2E1+/+ mice). After TCE exposure, the CYP2E1 +/+ had a similar increase in
4   PPARα mRNA (~ 2.3-fold) at both 1000 ppm and 2000 ppm TCE. Thus, without the presence
5   of CYP2E1 there did not appear to be increased PPARα mRNA expression. For PPARα protein
6   expression, there was a similar pattern with ~ 1.6-fold of control levels of protein in the CYP2E1
7   -/- mice after both 1000 ppm and 2000 ppm TCE exposures. In the CYP2E1 +/+ mice the
8   control level of PPARα protein was reported to be ~ 1.5-fold of the CYP2E1-/- control level.
9   Thus, while the mRNA expression was less, the protein level was greater. After TCE treatment
10 there was a 2.9-fold of control level of protein at 1000 ppm TCE and a 3.1-fold of control level
11 of protein at 2000 ppm. Thus, the magnitude of mRNA increase was similar to that of protein
12 expression for PPARα in CYP2E1 +/+ mice. The magnitude of either was 3-fold or less over
13 control after TCE exposure. This pattern was similar to that of TCA concentration formed in the
14 liver where there was very little difference between the 1000 and 2000 ppm exposure groups in
15 CYP2E1 +/+ mice. However, this pattern was not consistent with the liver enzyme and
16 histopathology of the liver that showed a much greater response after 2000 ppm exposure than
17 1000 ppm TCE. In addition, where the mean enzyme markers of liver injury and individual
18 animals displayed marked heterogeneity in response to TCE exposure, there was a much smaller
19 degree of variability in the mean mRNA expression and protein levels of PPARα
20
21   For peroxisomal bifunctional protein there was a greater increase after 1000 ppm TCE-
22 treated exposure than after 2000 ppm TCE-treatment for both the CYP2E1 -/- and CYP2E1 +/+
23 mice (i.e., there was a 2:1 ratio of mRNA expression in the 1000 ppm vs. 2000 ppm exposed
24 groups). The CYP2E1 +/+ mice had a much greater response than the CYP2E1-/- mice (i.e., the
25 CYP2E1-/- mice had a 2-fold of control and the CYP2E1+/+ mice had a 7.8-fold of control level
26 after 1000 ppm TCE treatment). For peroxisomal bifunctional protein expression the magnitude
27 of protein induction after TCE exposure was much greater than the magnitude of increase in
28 mRNA expression. In the CYP2E1-/- mice 1000 ppm TCE exposure resulted in a 6.9-fold of
29 control level of protein while the 2000 ppm TCE group had a 2.3-fold level. CYP2E1 +/+ mice
30 had a ~ 50% higher control level than CYP2E1 mice and after TCE exposure the level of
31 peroxisomal bifunctional protein expression was 44-fold of control at 1000 ppm TCE and 40-
32 fold of control at 2000 ppm.   Thus, CYP2E1-/- mice were reported to have less mRNA
33 expression and peroxisomal bifunctional protein formed than CYP2E1+/+ mice after TCE
34 exposure. However, there appeared to be more mRNA expression after 1000 ppm than 2000
35 ppm TCE in both groups and protein expression in the CYP2E1-/- mice. After 2000 ppm TCE,
36 there was similar peroxisomal bifunctional protein expression between the 1000 ppm and 2000
37 ppm TCE treated CYP2E1+/+ mice. Again this pattern was more similar to that of TCA
38 detection in the urine but not that of liver injury.
39
40   For VLCAD the expression of mRNA was similar between control and treated CYP2E1-
41 /- mice. For CYP2E1+/+ mice the control level of VLCAD mRNA expression was half that of
42 the CYP2E1-/- mice. After 1000 ppm TCE the mRNA level was 3.7-fold of control and after
43 2000 ppm TCE the mRNA level was 3.1-fold of control. For VLCAD protein expression was

1   1.8-fold of control after 1000 ppm and 1.6-fold of control after 2000 ppm in CYP2E1-/- mice.
2   The control level of VLCAD protein in CYP2E1+/+ mice appeared to be 1.2-fold control
3   CYP2E1-/- mice   After 1000 ppm TCE treatment the CYP2E1 -/- mice were reported to have
4   3.8-fold of control VLCAD protein levels and after 2000 ppm TCE treatment to have 3.9-fold of
5   control protein levels. Thus, although showing no increase in mRNA there was an increase in
6   VLCAD protein levels that was similar between the two TCE exposure groups in CYP2E1-/-
7   mice. Both VLCAD mRNA and protein levels were greater in CYP2E1 +/+ mice than CYP2E1-
8   /- mice after TCE exposure. This was not the case for peroxisomal bifunctional protein. The
9   magnitudes of TCE-induced increases in mRNA and protein increases were similar between the
10   1000 ppm and 2000 ppm TCE exposure concentrations, a pattern more similar to TCA detection
11   in the urine but not that of liver injury.
12
13   Finally, for CYP4A10 mRNA expression, there was an increase in expression after TCE
14   treatment of 3-fold for 1000 ppm and 5-fold after 2000 ppm in CYP2E1-/- mice. Thus, although
15   the enzyme assumed to be primarily responsible for TCE metabolism to TCA was missing, there
16   was still a response for the mRNA of this enzyme commonly associated with PPARα activation.
17   Of note is that urinary concentrations of TCA were not zero after TCE exposure in CYP2E1-/-
18   mice. Both 1000 and 2000 ppm TCE exposure resulted in ~ 0.44 mg TCA after 1 day or about
19   15 – 22% of that observed in CYP2E1+/+ mice. Thus, some metabolism of TCE to TCA is
20   taking place in the null mice, albeit at a reduced rate. For CYP2E1+/+ mice, 1000 ppm TCE
21   resulted in an 8.3-fold of control level of CYP4A10 mRNA and 2000 ppm TCE resulted in a 9.3-
22   fold of control level. The authors did not perform an analysis of CYP4A10 protein. The authors
23   state that "in particular, the mRNA levels of microsomal enzyme CYP4A10 significantly
24   increased in CYP2E1+/+ mice after TCE exposure in a dose-dependent manner." However, the
25   2-fold difference in TCE exposure concentrations did not result in a similar difference in
26   response as shown above. Both resulted in ~ 9-fold of control response in CYP2E1+/+ mice. As
27   with PPARα, peroxisomal bifunctional protein, and VLCAD, the response was more similar to
28   that of TCA detection in the urine and not measured of hepatic toxicity. These data are CYP2E1
29   metabolism of TCE is important in the manifestation of TCE liver toxicity, however it also
30   suggests that effects other than TCA concentration and indicators of PPARα are responsible for
31   acute hepatotoxicity resulting from very high concentrations of TCE.
32
33   The NFκB family and IκBα was also examined for mRNA and protein expression. These
34   cell signaling molecules are involved in inflammation and carcinogenesis and are discussed in
35   Section 3.3.3.3. and 3.4.1.4. Given that presence of hepatocellular necrosis in some of the
36   CYP2E1+/+ mice to varying degrees, inflammatory cytokines and cell signaling pathways would
37   be expected to be activated. The authors reported that "overall, TCE exposure did not
38   significantly increase the expression of p65 and p50 mRNAs in either CYP2E1+/+ or CYP2E1 -
39   /- mice… However, p52 mRNA expression significantly increased in the 2000 ppm group of
40   CYP2E1+/+ mice, and correlation analysis showed that a significant positive relationship existed
41   between the expression of NFκB p52 mRNA and plasma ALT activity.., while no correlation
42   was seen between NFκB p64 or p50 and ALT activity (data not shown)." The authors also note

1   that TCE treatments "did not increase the expression of TNFR1 and TNFR2 mRNA in
2   CYP2E1+/+ and CYP2E1 -/- mice (data not shown)."
3
4   A more detailed examination of the data reveals that there was a similar increases in p65,
5   p50, and p52 mRNA expression increases with TCE treatment in CYP2E1+/+ mice at both TCE
6   exposure levels. However, only p52 levels for the 2000 ppm-exposed mice were reported to be
7   statistically significant (see comment above about the statistical power of the experimental
8   design and variability between animals). For 1000 ppm TCE exposure the levels of p65, p50,
9   and p52 mRNA expression were 1.5-fold, 1.8-fold, and 2.0-fold of control. For 2000 ppm TCE
10   the levels of p65, p50, and p52 mRNA expression were 1.8-fold, 1.8-fold, and 2.1-fold of
11   control. Thus, there was generally a similar response in all of these indicators of NFκB mRNA
12   expression in CYP2E1 +/+ mice that was mild with little to no difference between the 1000 ppm
13   and 2000 ppm TCE exposure levels. For IκBα mRNA expression there was not difference
14   between control and treatment groups for either type of mice. For CYP2E1-/- mice there
15   appeared to be a ~ 50% decrease in P52 mRNA expression in mice treated with both exposure
16   concentrations of TCE. The authors plotted the relationship between p52 mRNA and plasma
17   ALT concentration for both CYP2E1 -/- and CYP2E1 +/+ mice together and claimed the
18   correlation coefficient (r= 0.5075) was significant. However, of note is that none of the CYP2E1
19   -/- mice were reported to have either hepatic necrosis or significant increases in ALT detection.
20
21   For protein expression the authors showed results for p50 and p42 proteins. The control
22   CYP2E1-/- mice appeared to have a slightly lower level of p50 protein expression (~ 30%) with
23   a much larger increase in p52 protein expression (i.e., 2.1-fold) than CYP2E1+/+ mice. There
24   appeared to be a 2-fold increase in p50 protein expression after both 1000 ppm and 2000 ppm
25   TCE exposures in the CYP2E1 +/+ mice and a similar increase in p52 protein levels (i.e., 1.9-
26   fold and 2.5-fold of control for 1000 ppm and 2000 ppm TCE exposures, respectively). Thus,
27   the magnitude of mRNA and protein levels were similar for p50 and p52 in CYP2E1 +/+ mice
28   and there was no difference between the 1000 ppm and 2000 ppm treatments. For the CYP2E1 -
29   /- mice there was a modest increase in p50 protein after TCE exposure (1.1-fold and 1.3-fold of
30   control for 1000 ppm and 2000 ppm respectively) and a slight decrease in p52 protein (0.76-fold
31   and 0.79-fold of control). There was little evidence that the patterns of either expression or
32   protein production of NFκB family and IκBα corresponded to the markers of hepatic toxicity or
33   that they exhibited a dose-response. The authors note that although he expression of p50 protein
34   increased in CYP2E1+/+ mice, "the relationship between p50 protein and ALT levels was not
35   significant (data not shown)." For TNFR1 there appeared to be less protein expression in the
36   CYP2E1 +/+ mice than the CYP2E1-/- mice (i.e., the null mice levels were 1.8-fold of the wild-
37   type mice levels). Treatment with TCE resulted in mild decrease of protein levels in the
38   CYP2E1 -/- mice and a 1.4-fold and 1.7-fold of control level in the CYP2E1+/+ mice for 1000
39   ppm and 2000 ppm levels, respectively. For p65, although TCE treatment–related effects were
40   reported, of note the levels of protein were 2.4 higher in the CYP2E1+/+ mice than the CYP2E1-
41   /- mice. Thus, protein levels of the NFκB family appeared to have been altered in the knockout
42   mice. Also, as noted in Section 3.4.1.4, the origin of the NF-κB is crucial as to its effect in the
43   liver and the results of this report are for whole liver homogenates that contain parenchymal as

1 well as nonparenchymal cell and have been drawn from liver that are heterogeneous in the
2 magnitude of hepatic necrosis. The authors suggest that "TCA may act as a defense against
3 hepatotoxicity cause by TCE-delivered reactive metabolite(s) via PPARα in CYP2E1+/+ mice."
4 However, the data from this do not support such an assertion.
5
6 2.2. Subchronic and Chronic Studies of TCE
7
8   For the purposes of this discussion, studies of duration of 4 weeks or more are considered
9 subchronic. Like those of shorter duration, there is variation in the depth of study of liver
10 changes induced by TCE with many of the longer duration studies focused on the induction of
11 liver cancer. Many subchronic studies were conducted a high doses of TCE that caused toxicity
12 with limited reporting of effects. Similar to acute studies some of the subchronic and chronic
13 studies have detailed examinations of the TCE-induced liver effects while others have reported
14 primarily liver weight changes as a marker of TCE-response. Similar issues also arise with the
15 impact of differences in initial and final body weights between control and treatment groups on
16 the interpretation of liver weight gain as a measure of TCE-response. For many of the
17 subchronic inhalation studies, issues associated with whole body exposures make determination
18 of dose levels difficult. For gavage experiments, death from gavage dosing, especially at higher
19 TCE exposures, is a recurring problem and, unlike inhalation exposures, the effects of vehicle
20 can also be at issue for background liver effects. Concerns regarding effects of oil vehicles,
21 especially corn oil, have been raised with Kim et al. (1990) noting that a large oil bolus will not
22 only produce physiological effects, but alter the absorption, target organ dose, and toxicity of
23 VOCs. Charbonneau et al. (1991) reported that corn oil potentiates liver toxicity from acetone
24 administration that is not related to differences in acetone concentration. Several oral studies in
25 particular document that use of corn oil gavage induces a different pattern of toxicity, especially
26 in male rodents (see Merrick et al., 1989, Section 2.2.1. below). Several studies listed below
27 report the effects of hepatocellular DNA synthesis and indices of lipid peroxidation (i.e., Channel
28 et al., 1998) are especially subject to background vehicle effects. Rusyn et al (1999) report that a
29 single dose of dietary corn oil increases hepatocyte DNA synthesis 24 hours after treatment by ~
30 3.5 fold, activation of NF-κB to a similar extent ~ 2 hours after treatment almost exclusively in
31 Kupffer cells, a ~ 3-4 fold increase in hepatocytes after 8 hours, and increased in TNFα mRNA
32 between 8 and 24 hours after a single dose in female rats. In regard to studies that have used the
33 i.p. route of administration, as noted by Kawamoto et al (1988) (see Section 2.2.10 below),
34 injection of TCE may result in paralytic ileus and peritonitis and that subcutaneous treatment
35 paradigm will result in TCE not immediately being metabolized but retained in the fatty tissue.
36 Wang and Stacey (1990) state that "intraperitoneal injection is not particularly relevant to
37 humans" and that intestinal interactions require consideration in responses such as increase
38 serum bile acid (see Section 2.3.5 below).
39
40 2.2.1. Merrick et al. 1989
41
42   The focus of this study was the examination of potential differences in toxicity or orally
43 gavaged TCE administered in corn oil an aqueous vehicle in B6C3F1 mice. As reported by

1 Melnick et al. (1987) above, corn oil administration appeared to have an effect on peroxisomal
2 enzyme induction. TCE (99.5% purity) was administered in corn oil or an aqueous solution of
3 20% Emulphor to 14-17 week old mice (n = 12/group) at 0, 600, 1200 and 2400 mg/kg/day
4 (males) and 0, 450, 900, and 1800 mg/kg/day (females) 5 times a week for 4 weeks. The authors
5 state that due to "varying lethality in the study, 10 animals per dose group were randomly
6 selected (where possible) among survivors for histological analysis." Hepatocellular lesions
7 were characterized "as a collection of approximately 3-5 necrotic hepatocytes surrounded by
8 macrophages and polymorphonuclear cells and histopathological grading was reported as based
9 on the number of necrotic lesions observed in the tissue sections: 0 = normal; 1 = isolated lesions
10 scattered throughout the section; 2 = one to five scattered clusters of necrotic lesions; 3 = more
11 than five scattered clusters of necrotic lesions; and 4 = clusters of necrotic lesions observed
12 throughout the entire section." The authors described lipid scoring of each histological section
13 as "0 = no Oil-Red O staining present; 1 = less than 10% staining; 2 = 10-25% staining; 3 = 25-
14 30% staining; and 4 = greater than 50% staining."
15
16   The authors reported dose-related increases in lethality in both males and females
17 exposed to TCE in Emulphor with all male animals dying at 2400 mg/kg/day with 8/12 females
18 dying at 1800 mg/kg/day. In both males and females, 2/12 animals also died at the next highest
19 dose as well with no unscheduled deaths in control or lowest dose animals. For corn oil gavaged
20 mice, there were 1-2 animals in each TCE treatment groups of male mice that died while there
21 were no unscheduled deaths in female mice. The authors state that lethality occurred within the
22 first week after chemical exposure. The authors present data for final body weight and
23 liver/body weight values for 4 weeks of exposure and list the number of animals per group to be
24 10-12 for corn oil gavaged animals and the reduced number of animals in the Emulphor gavaged
25 animals reflective of lethality and limiting the usefulness of this measure at the highest doses
26 (i.e., 1800 mg/kg/day for female mice). In mice treated with TCE in Emulphor gavage, the final
27 body weight of control male animals appeared to be lower than those which were treated with
28 TCE while for female mice the final body weights were similar between treated and control
29 groups. For male mice treated with Emulphor, body weights were 22.8 ± 0.8, 25.3 ± 0.5, and
30 24.3 ± 0.4 g for control, 600 mg/kg/day, and 1200 mg/kg/day and for female mice body weights
31 were 20.7 ± 0.4, 21.4 ± 0.3, and 20.5 ± 0.3 g for control, 450 mg/kg/day, and 900 mg/kg/day of
32 TCE.
33
34   For % liver/body weight ratios, male mice were reported to have 5.6 ± 0.2, 6.6 ± 0.1, and
35 7.2 ± 0.2 % for control, 600 mg/kg/day, and 1200 mg/kg/day and for female mice were 5.1 ± 0.1,
36 5.8 ± 0.1, and 6.5 ± 0.2 % for control, 450 mg/kg/day, and 900 mg/kg/day of TCE. These values
37 represent 1.11-fold and 1.07-fold of control for final body weight in males exposed to 600 and
38 1200 mg/kg/day and 1.18-fold and 1.29-fold of control for % liver/body weight, respectively.
39 For females, they represent 1.04-fold and 0.99-fold of control for final body weights in female
40 exposed to 450mg/kg/day and 900 mg/kg/day and 1.14-fold and 1.27-fold of control for %
41 liver/body weight, respectively.

1   In mice treated with corn oil gavage the final body weight of control male mice was
2   similar to the TCE treatment groups and higher than the control value for male mice given
3   Emulphor vehicle (i.e., 22.8 ± 0.8 g for Emulphor control vs. 24.3 ± 0.6 g for corn oil gavage
4   controls or a difference of $\sim 7\%$). The final body weights of female mice were reported to be
5   similar between the vehicles and TCE treatment groups. The baseline % liver/body weight was
6   also lower for the corn oil gavage control male mice (i.e., 5.6 % for Emulphor vs. 4.7 % for corn
7   oil gavage or a difference of $\sim 19\%$ that was statistically significant). Although the final body
8   weights were similar in the female control groups, the % liver/body weight was greater in the
9   Emulphor vehicle group (5.1 ± 0.1 % in Emulphor vehicle group vs. 4.7 ± 0.1 % for corn oil
10   gavage or a difference of ~ 9 % that was statistically significant). For male mice treated with
11   corn oil, final body weights were 24.3 ± 0.6, 24.3 ± 0.4, 25.2 ± 0.6 and 25.4 ± 0.5 g for control,
12   600 mg/kg/day, 1200 mg/kg/day and 2400 mg/kg/day, and for female mice body weights were
13   20.2 ± 0.3, 20.8 ± 0.5, 21.8 ± 0.3 g, and 22.6 ± 0.3 g for control, 450 mg/kg/day, 900 mg/kg/day,
14   and 1800 mg/kg/day of TCE. For % liver/body weight ratios, male mice were reported to have
15   4.7 ± 0.1, 6.4 ± 0.1, 7.7 ± 0.1 % , and 8.5 ± 0.2 % for control, 600 mg/kg/day, 1200 mg/kg/day,
16   and 2400 mg/kg/day and for female mice were 4.7 ± 0.1, 5.5 ± 0.1, 6.0 ± 0.2 %, and 7.2 ± 0.1 %
17   for control, 450 mg/kg/day, 900 mg/kg/day, and 1800 mg/kg/day of TCE. These values
18   represent 1.0-fold, 1.04-fold, and 1.04-fold of control for final body weight in males exposed to
19   600, 1200 mg/kg/day and 2400 mg/kg/day TCE and 1.36-fold, 1.64-fold, and 1.81-fold of
20   control for % liver/body weight, respectively. For females they represent 1.03-fold, 1.08-fold,
21   and 1.12-fold of control for body weight in female exposed to 450 mg/kg/day, 900 mg/kg/day,
22   and 1800 mg/kg/day and 1.17-fold, 1.28-fold, and 1.53-fold of control for % liver/body weight,
23   respectively.
24
25   Because of premature mortality, the difference in TCE treatment between the highest
26   doses that are vehicle-related cannot be determined. The decreased final body weight and
27   increased % liver/body weight ratios in the Emulphor control animals make comparisons of the
28   exact magnitude of change in these parameters due to TCE exposure difficult to determine as
29   well as differences between the vehicles. The authors did not present data for age-matched
30   controls which did not receive vehicle so that the effects of the vehicles cannot be determined
31   (i.e., which vehicle control values were most similar to untreated controls given that there was a
32   difference between the vehicle controls). A comparison the % liver/body weight ratios at
33   comparable doses between the two vehicles shows little difference in TCE-induced liver weight
34   increases in female mice. However, the corn oil vehicle group was reported to have a greater
35   increase in comparison to controls for male mice treated with TCE at the two lower dosage
36   groups. Given that the control values were approximately 19% higher for the Emulphor group,
37   the apparent differences in TCE-dose response may have reflected the differences in the control
38   values rather than TCE exposure. Because controls without vehicle were not examined, it cannot
39   be determined whether the difference in control values was due to vehicle administration or
40   whether a smaller or younger group of animals was studied on one of the control groups. The
41   body weight of the animals was also not reported by the authors at the beginning of the study so
42   that the impact of initial differences between groups vs. treatment cannot be accurately
43   determined.

1   Serum enzyme activities for ALT, AST and LDH (markers of liver toxicity) showed that
2   there was no difference between vehicle groups at comparable TCE exposure levels for male or
3   female mice. Enzyme levels appeared to be elevated in male mice at the higher doses (i.e., 1200
4   and 2400 mg/kg/day for ALT and 2400 mg/kg/day for AST) with corn oil gavage inducing
5   similar increases in LDH levels at 600 mg/kg/day, 1200 mg/kg/day and 2400 mg/kg/day TCE.
6   For ALT and AST there appeared to be a dose-related increase in male mice with the 2400
7   mg/kg treatment group having much greater levels than the 1200 mg/kg group. In Emulphor
8   treatment groups there was a similar increase in ALT levels in males treated with 1200 mg/kg
9   TCE as with those treated with corn oil and those increases were significantly elevated over
10   control levels. For LDH levels there were similar increase at 1200 mg/kg TCE for male mice
11   treated using either Emulphor or corn oil.   The authors report that visible necrosis was observed
12   in 30-40% of male mice administered TCE in corn oil but not that there did not appear to be a
13   dose-response (i.e., the score for severity of necrosis was reported to be 0, 4, 3, and 4 for corn oil
14   control, 600 mg/kg/day, 1200 mg/kg/day, and 2400 mg/kg/day treatment groups from 10 male
15   mice in each group).   No information in regard to variation between animals was given by the
16   authors. For male mice given Emulphor gavage the extent of necrosis was reported to be 0, 0,
17   and 1 for 0, 600 mg/kg/day and 1200 mg/kg/day TCE exposure, respectively. For female mice,
18   the extent of necrosis was reported to be 0 for all control and TCE treatment groups using either
19   vehicle. Thus, except for LDH levels in male mice exposed to TCE in corn oil there was not a
20   correlation with the extent of necrosis and the increases in ALT and AST enzyme levels.
21   Similarly there was an increase in ALT levels in male mice treated with 1200 mg/kg/day
22   exposure to TCE in Emulphor that did not correspond to increased necrosis. For Oil-Red O
23   staining there was a score of 2 in the Emulphor treated control male and female mice while 600
24   mg/kg/day TCE exposure in Emulphor gavaged male mice and 900 mg/kg/day TCE in corn oil
25   gavaged female mice had a score of 0, along with the corn oil gavage controls in male mice. For
26   female control mice treated with corn oil gavage, the staining was reported to have a score of 3.
27   Thus, there did not appear to be a dose-response in Oil-Red oil staining although the authors
28   claimed there appeared to be a dose-related increase with TCE exposure.   The authors described
29   lesions produced by TCE exposure as "focal and were surrounded by normal parenchymal tissue.
30   Necrotic areas were not localized in any particular regions of the lobule. Lesions consisted of
31   central necrotic cells encompassed by hepatocytes with dark eosinophilic staining cytoplasm
32   which progressed to normal-appearing cells. Areas of necrosis were accompanied by localized
33   inflammation consisting of macrophages and polymorphonuclear cells." No specific
34   descriptions of histopathology of mice given Emulphor were provided in terms of effects of the
35   vehicle or TCE treatment. The scores for necrosis was reported to be only a 1 for the 1200
36   mg/kg concentration of TCE in male mice gavaged with Emulphor but 3 for male mice given the
37   same concentration of TCE in corn oil. However enzyme levels of ALT, AST, and LDH were
38   similarly elevated in both treatment groups.

39

40   These results do indicate that administration of TCE for 4 weeks via gavage using
41   Emulphor resulted in mortality of all of the male mice and most of the female mice at a dose in
42   corn oil that resulted in few deaths. Not only was there a difference in mortality, but vehicle also
43   affected the extent of necrosis and enzyme release in the liver (i.e, Emulphor vehicle caused

1 mortality as the highest dose of TCE in male and female mice that was not apparent from corn
2 oil gavage, but Emulphor and TCE exposure induced little if any focal necrosis in males at
3 concentrations of TCE in corn oil gavage that caused significant focal necrosis).   In regard to
4 liver weight and body weight changes, TCE exposure in both vehicles at non-lethal doses
5 induced increased % liver/body weight changes male and female mice that increased with TCE
6 exposure level. The difference in baseline control levels between the two vehicle groups
7 (especially in males) make a determination of the quantitative difference vehicle had on liver
8 weight gain problematic although the extent of liver weight increase appeared to be similar
9 between male and female mice given TCE via Emulphor and female mice given TCE via corn
10 oil. In general, enzymatic markers of liver toxicity and results for focal hepatocellular necrosis
11 were not consistent and did not reflect dose-responses in liver weight increases. The extent of
12 necrosis did not correlate with liver weight increases and was not elevated by TCE treatment in
13 female mice treated with TCE in either vehicle, or in male mice treated with Emulphor. There
14 was a reported difference in the extent of necrosis in male mice given TCE via corn oil and
15 female mice given TCE via corn oil but the necrosis did not appear to have a dose-response in
16 male mice. Female mice given corn oil and male and female mice given TCE in Emulphor had
17 no to negligible necrosis although they had increased liver weight from TCE exposure.
18
19 ### 2.2.2. Goel et al. 1992
20
21   The focus of this study was the description of TCE exposure related changes in mice after
22 28 days of exposure with regard to TCE-induced pathological and liver weight change. Male
23 Swiss mice (20-22 g body weight or 9% difference) were exposed to 0, 500, 1000 or 2000
24 mg/kg/day TCE (BDH analytical grade) by gavage in groundnut oil (n= 6 per group) 5 days a
25 week for 28 days. The ages of the mice were not given by the authors. Livers were examined
26 for "free -SH contents", total proteins, catalase activity, acid phosphatase activity, and "protein
27 specific for peroxisomal origin of approx, 80 kd."   The authors report no statistically significant
28 change in body weight with TCE treatment but a significant increase in liver weight. Body
29 weight (mean ± SE) was reported to be 32.67 ± 1.54, 31.67 ± 0.61, 33.00 ± 1.48, and 27.80 ±
30 1.65 g from exposure to oil control, 500, 1000, and 2000 mg/kg/day TCE, respectively. There
31 was a 15% decrease in body weight at the highest exposure concentration of TCE that was not
32 statistically significant, but the low number of animals examined limits the power to detect a
33 significant change.   The % relative liver/ body weight was reported to be 5.29 ± 0.48, 7.00 ±
34 0.36, 7.40 ± 0.39, and 7.30 ± 0.48 % from exposure to oil control, 500, 1000, and 2000
35 mg/kg/day TCE, respectively. This represents 1.32-fold, 1.41-fold, and 1.38-fold of control in %
36 liver/body weight for 500, 1000, and 2000 mg/kg/day TCE, respectively. The "free –SH
37 content" in μmole –SH/g tissue was reported to be 5.47 ± 0.17, 7.46 ± 0.21, 7.84 ± 0.34, and
38 7.10 ± 0.34 from exposure to oil control, 500, 1000, and 2000 mg/kg/day TCE, respectively.
39 This represents 1.37-fold, 1.44-fold, and 1.30-fold of control in –SH/g tissue weight for 500,
40 1000, and 2000 mg/kg/day TCE, respectively. Total protein content in the liver in mg/g tissue
41 was reported to be 170 ± 3, 183 ± 5, 192 ± 7, and 188 ± 3 from exposure to oil control, 500,
42 1000, and 2000 mg/kg/day TCE, respectively. This represents 1.08-fold, 1.13-fold, and 1.11-
43 fold of control in total protein content for 500, 1000, and 2000 mg/kg/day TCE, respectively.

1   Thus, the increases in liver weight, "free -SH content" and increase protein content were
2   generally parallel and all suggest that liver weight increases had reached a plateau at the 1000
3   mg/kg/day exposure concentration perhaps reflecting toxicity at the highest dose as demonstrated
4   by decreased body weight in this study.
5
6   The enzyme activities of δ-ALA dehydrogenase ("a key enzyme in heme biosynthesis"),
7   catalase, and acid phosphatase were assayed in liver homogenates. Treatment with TCE
8   decreased δ-ALA dehydrogenase activity to a similar extent at all exposure levels (32 − 35%
9   reduction). For catalase the activity as units of catalase/mg protein was reported to be 25.01 ±
10   1.81, 32.46 ± 2.59, 41.11 ± 5.37, and 33.96 ± 3.00 from exposure to oil control, 500, 1000, and
11   2000 mg/kg/day TCE, respectively. This represents 1.30-fold, 1.64-fold, and 1.36-fold in
12   catalase activity for 500, 1000, and 2000 mg/kg/day TCE, respectively. The increasing
13   variability in response with TCE exposure concentration is readily apparent from this data as is
14   the decrease at the highest dose, perhaps reflective of toxicity. For acid phosphatase activity in
15   the liver there was a slight increase (5-11%) with TCE exposure that did not appear to be dose-
16   related.
17
18   The authors report that histologically, "the liver exhibits swelling, vacuolization,
19   widespread degeneration/necrosis of hepatocytes as well as marked proliferation of endothelial
20   cells of hepatic sinusoids at 1000 and 2000 mg/kg TCE doses."   Only one figure is given at the
21   light microscopic level in which it is impossible to distinguish endothelial cells from Kupffer
22   cells and no quantitative measures or proliferation were examined or reported to support the
23   conclusion that endothelial cells are proliferating in response to TCE treatment. Similarly, no
24   quantitation regarding the extent or location of hepatocellular necrosis is given. The presence or
25   absence of inflammatory cells is not noted by the authors as well. In terms of white blood cell
26   count, the authors note that it is slightly increased at 500 mg/kg/day but decreased at 1000 and
27   2000 mg/kg/day TCE, perhaps indicating macrophage recruitment from blood to liver and
28   kidney, which was also noted to have pathology at these concentrations of TCE.
29
30   **2.2.3. Kjellstrand et al. 1981**
31
32   This study was conducted in mice, rats, and gerbils and focused on the effects of 150 ppm
33   TCE exposure via inhalation on body and organ weight. No other endpoints other than organ
34   weights were examined in this study and the design of the study is such that quantitative
35   determinations of the magnitude of TCE response are very limited. NMRI mice (weighing ~ 30
36   g with age not given), Sprague-Dawley rats (weighing ~ 200 g with age not given, and
37   Mongolian gerbils (weighing ~ 60 g with age not given) were exposed to 150 ppm TCE
38   continuously. Mice were exposed for 2, 5, 9, 16, and 30 days with the number of exposed
39   animals and controls in the 2, 5, 9 and 16 days groups being 10. For 30-day treatments there
40   were 2 groups of mice containing 20 mice per group and one group containing 12 mice per
41   group.   In addition there was a group of mice (n= 15) exposed to TCE for 30 days and then
42   examined 5 days after cessation of exposure and another group (n= 20) exposed to TCE for 30
43   days and then examined 30 days after cessation of exposure. For rats there were 3 groups

1   exposed to TCE for 30 days which contained 24, 12 and 10 animals per group. For gerbils there
2   were 3 groups exposed to TCE for 30 days which contained 24, 8 and 8 animals per group. The
3   groups were reported to consist of equal numbers of males and female but for the mice exposed
4   to TCE for 30 days and then examined 5 days later, the number was 10 males and 5 females.
5   Body weights were reported to be recorded before and after the exposure period. However the
6   authors state "for technical reasons the animals within a group were not individually identified,
7   i.e., we did not know which initial weight in the group corresponded to which final one." They
8   authors state that this design presented problems in assessing the precision of the estimate. They
9   go on to state that rats and gerbils were partially identifiable as the animals were housed 3 to a
10 cage and cage averages could be estimated. Not only were mice in one group housed together
11 but "even worse: at the start of the experiment, the mice in M2 [sic, group exposed for 2 days]
12 and M9 [sic group exposed for 9 days] were housed together, and similarly M5 [group exposed
13 for 5 days] and M16 [sic group exposed for 16 days]. Thus, we had, e.g., 10 initial weights for
14 exposed female mice in M2 and M9 where we could not identify those 5 which were M2
15 weights. Owing to this bad design (forced upon us by the lack of exposure units), we could not
16 study weight gains for mice and so we had to make do with an analysis of final weights." The
17 problems with the design of this study are obvious from the description given by the authors
18 themselves. The authors state that they assumed that the larger the animal the larger the weight
19 of its organs so that all organ weights were converted into relative weights as percentage of body
20 weight. The fallacy of this assumption is obvious, especially if there was toxicity that decreased
21 body weight and body fat but at the same time caused increased liver weight as has been
22 observed in many studies at higher doses of TCE. In fact, Kjellstrand et al. (1983b) report that a
23 150 ppm TCE exposure for 30 days does significantly decreases body weight while elevating
24 liver weight in a group of 10 male NMRI mice. Thus, the body weight estimates from this study
25 are inappropriate for comparison to those in studies where body weights were actually measured.
26 The liver/body weight ratios that would be derived from such estimates of body weights would
27 be meaningless.   The group averages for body weight reported for female mice at the beginning
28 of the 30 – day exposure varied significantly and ranged from 23.2 to 30.2 g (~ 24%).   For
29 males the group averages ranged from 27.3 to 31.4 g (~ 14%).   For male mice there was no
30 weight estimate for the animals that were exposed for 30 days and then examined 30 days after
31 cessation of exposure.
32
33   The authors only report relative organ weight at the end of the experiment rather than the
34 liver weights for individual animals.   Thus, these values represent extrapolations based on to
35 what body weight may have been. For mice that were exposed to TCE for 30 days and the
36 examined after 30 days of exposure, male mice were reported to have "relative organ weight" for
37 liver of 4.70 ± 0.10 % vs. 4.27 ± 0.13 % for controls. However, there were no initial body
38 weights reported for these male mice and the body weights are extrapolated values. Female mice
39 exposed for 30 days and then examined 30 days after cessation of exposure were reported to
40 have "relative organ weights" for liver of 4.42 ± 0.11 % vs. 3.62 ± 0.09 %. The group average of
41 initial body weights for this group was reported by the authors. Although the initial body weight
42 for female control mice as a group average was reported to be similar between the female group
43 exposed to 30 days of TCE and sacrificed 30 days later and those exposed for 30 days and

1   sacrificed 5 days later (30.0 g vs. 30.8 g), the liver/body weight ratio varied significantly in
2   these controls (4.25 ± 0.19 vs. 3.62 ± 0.09) as did the number of animals studied (5 female mice
3   in the animals sacrificed after 5 days exposure vs. 10 female mice in the group sacrificed after 30
4   days exposure). In addition, although there were differences between the 3 groups of mice
5   exposed to TCE for 30 days and then sacrificed immediately, the authors present the data for
6   extrapolated liver/body weight as pooled results between the 3 groups. In comparison to control
7   values, the authors report 1.14-fold, 1.35-fold, 1.58-fold, 1.47-fold, and 1.75-fold of control for
8   % liver/body weight using body weight extrapolated values in male mice at 2, 5, 9, 16, and 30
9   days of TCE exposure, respectively. For females they report 1.27-fold, 1.28-fold, 1.49-fold,
10 1.41-fold, and 1.74-fold of control at 2, 5, 9, 16, and 30 days of TCE, respectively. Although the
11 authors combine female and male relative increases in liver weight in a figure, assign error bars
12 around these data point, and attempt to draw assign a time-response curve to it, it is clear from
13 this data, especially for female mice, do not display time-dependent increase in liver/body weight
14 from 5 to 16 days of exposure and that a comparison of results between 5 animals and 26 is very
15 limited in interpretation. Of note is the wide variation in the control values for relative
16 liver/body weight. For male mice there did not seem to be a consistent pattern with increasing
17 duration of the experiment with values at 4.61, 5.15, 5.05, 4.93, and 4.04 % for 2, 5, 9, 16, and
18 30 day exposure groups. This represented a difference of ~ 27%. For female mice the relative
19 liver/body weight was 4.14, 4.58, 4.61, 4.70, and 3.99 % for 2, 5, 9, 16, and 30 day exposure
20 groups. Thus, it appears that the average relative liver/body weight % was higher in the 5, 9, and
21 16 day treatment group for both genders than that to the 30 day group and was consistent
22 between these days. There is no apparent reason for there to be such large difference between 16
23 day and 30-day treatment groups due to increasing age of the animals. Of note is that for the
24 control groups pared with animals treated for 30 days and then examined 30 days later, the male
25 mice had increases in relative liver/body weight (4.27% vs. 4.04%) but that the females had a
26 decrease (3.62% vs. 3.99%). Such variation between controls does not appear to be age and size
27 related but to variations in measure or extrapolations, which can affect comparisons between
28 treated and untreated groups and add more uncertainty to the estimates.
29
30   The number of mice in the groups exposed to 2 though 16 days were only 5 animals for
31 each gender in each group while the number of animals reported in the 30 –day exposure group
32 numbered 26 for each gender.
33
34   For animals exposed to 30 days and then examined after 5 or 30 days, male mice were
35 reported to have % liver/body weight 1.26-fold and 1.10-fold of control after 5 and 30 days
36 cessation of exposure while female mice were reported to have values of 1.14-fold and 1.22-fold
37 of control after 5 and 30 days cessation of exposure, respectively. Again, the male mice exposed
38 for 30 days and then examined after 30 days of cessation of exposure did not have reported
39 initial body weights giving this value a great deal of uncertainty. Thus while liver weights
40 appeared to increase during 30 days of exposure to TCE and decreased after cessation of
41 exposure in both genders of mice, the magnitudes of the increases and decrease cannot be
42 determined from this experimental design. Of note is that liver weights appeared to still be
43 elevated after 30 days of cessation exposure.

1
2   In regard to initial weights, the authors report that the initial weight of the rats were
3   different in the 3 experiments they conducted with them and state that "in those 2 where
4   differences were found in females, their initial weights were about 200 g and 220 g, respectively,
5   while the corresponding weights were only about 160 g in that experiment where no differences
6   were found." The differences in initial body weight of the rat groups were significant. In
7   females group averages were 198, 158, and 224 g, for groups 1, 2, and 3, respectively and for
8   males group averages were 222, 166, and 248 g for groups 1,2, and 3 respectively. This
9   represents as much as a 50% difference in initial body weights between these TCE treatment
10   groups. Control values varied as well with group averages for controls ranging from167g for
11   group 2 to 246 g for group 3 at the start of exposure. For female rats control groups ranged from
12   158 to 219 g at the start of the experiment. The number of animals in each group varied greatly
13   as well making quantitative comparison even more difficult with the numbers varying between 5
14   and 12 for each gender in rats exposed for 30 days to TCE. The authors pooled the results for
15   these very disparate groups of rats in their reporting of relative organ weights. They reported
16   1.26-fold and 1.21-fold of control in male and female rat % relative liver/body weight after 30
17   days of TCE exposure. However, as stated above, these estimates are limited in their ability to
18   provide a quantitative estimate of liver weight increase due to TCE.
19
20   There were evidently differences between the groups of gerbils in response to TCE with
21   one group reported to have larger weight gain than control and the other 2 groups reported to not
22   show a difference by the authors. Of the 3 groups of gerbils, group 1 contained 12 animals per
23   gender but groups 2 and 3 only 4 animals per gender. As with the rat experiments, the initial
24   average weights for the groups varied significantly (30% in females and males). The authors
25   pooled the results for these very disparate groups of gerbils in their reporting of relative organ
26   weights as well. They reported a nearly identical increase in relative liver/body weight increase
27   for gerbil (1.22-fold of control value in males and 1.25-fold in females) as for the rat after 30
28   days of TCE exposure. However, similar caveats should be applied in the confidence in this
29   experimental design to determine the magnitudes of response to TCE exposure.
30
31   **2.2.4. Woolhiser et al. 2006.**
32
33   An unpublished report by Woolhiser et al (2006) was received by the EPA to fill the
34   "priority data needed" (PDN) for the immunotoxicity of TCE as identified by the Agency for
35   Toxic Substances and Disease Registry (ATSDR) and designed to satisfy U.S. EPA OPPTS
36   870.7800 Immunotoxicity Test Guidelines. The study was conducted on behalf of the
37   Halogenated Solvents Industry Alliance and has been submitted to the EPA but not published.
38   Although conducted as an immunotoxicity study, it does contain information regarding liver
39   weight increases in female Sprague Dawley (CD) female rats exposed to 0, 100 ppm, 300 ppm,
40   and 1000 ppm TCE for 6 hours/day, 5 days/week for 4 weeks. The rats were 7 weeks of age at
41   the start of the study. The report gives data for body weight and food weight for 16 animals per
42   exposure group and the mean body weights ranged between 181.8g to 185.5 g on the first day of
43   the experiment. Animals were weight pre-exposure, twice during the first week, and then "at

1 least weekly throughout the study." All rats were immunized with a single i.v. injection of
2 SRBC via the tail vein at day 25. Liver weights were taken and samples of liver retained "should
3 histopathological examinination have been deemed necessary." But histopathological analysis
4 was not conducted on the liver.
5
6   The effect on body weight gain by TCE inhalation exposure was shown by 5 days and
7 continued for 10 days of exposure in the 300 ppm and 1000 ppm exposed groups. By day 28, the
8 mean body weight for the control group was reported to be 245.7 g but 234.4 g, 232.4 g, and
9 232.4 g for the 100 ppm, 300 ppm, and 1000 ppm exposure groups, respectively. Food
10 consumption was reported to be decreased in the day1-5 measurement period for the 300 ppm
11 and 1000 ppm exposure groups and in the 5-10 day measurement period for the 100 ppm group.
12 Although body weight and food consumption data are available for 16 animals per exposure
13 group, for organ and organ/body weight summary data, the report gives information for only 8
14 rats per group. The report gives individual animal data in its appendix so that the data for the 8
15 animals in each group examined for organ weight changes could be examined separately. The
16 final body weights were reported to be 217.2 g, 212.4 g, 203.9 g, and 206.9 g for the control, 100
17 ppm, 300 ppm, and 1000 ppm exposure groups containing only 8 animals. For the 8-animal
18 exposure groups, the mean initial body weights were 186.6 g, 183.7 g, 181.6 g, and 181.9 g for
19 the control, 100 ppm, 300 ppm, and 1000 ppm exposure groups. Thus, there was a difference
20 from the initial and final body weight values given for the groups containing 16 rats and those
21 containing 8 rats. The ranges of initial body weights for the eight animals were 169.8 g – 204.3
22 g, 162.0 g – 191.2 g, 169.0g - 201.5 g, and 168.2 g – 193.7 g for the control, 100 ppm, 300 ppm,
23 and 1000 ppm groups. Thus, the control group began with a larger mean value and large range
24 of values (20% difference between highest and lowest weight rat) than the other groups.
25
26   In terms of the % liver/body weight ratios, an increase due to TCE exposure is reported in
27 female rats, although body weights were larger in the control group and the two higher exposure
28 groups did not gain body weight to the same extent as controls. The mean % liver/body weight
29 ratios were 3.23 %, 3.39 %, 3.44 %, and 3.65%, respectively for the control, 100 ppm, 300 ppm,
30 and 1000 ppm exposure groups. This represented 1.05-fold, 1.07-fold, and 1.13-fold of control
31 % liver/body weight changes in the 100 ppm, 300 ppm and 1000 ppm-exposure groups.
32 However, the small number of animals and the variation in initial animal weight limit the ability
33 of this study to determine statistically significant increases and the authors report that only the
34 1000 ppm group had statistically significant increased liver weight increases.
35
36 **2.2.5. Kjellstrand et al. 1983(a)**
37
38   This study examined seven strains of mice (wild, C57BL, DBA, B6CBA, A/sn, NZB and
39 NMRI) after continuous inhalation exposure to 150 ppm TCE for 30 days. "Wild" mice were
40 reported to be composed of "three different strains: 1. Hairless (HR) from the original strain, 2.
41 Swiss (outbred), and 3. Furtype Black Pelage (of unknown strain)." The authors do not state the
42 age of the animals prior to TCE exposure but state that weight-matched controls were exposed to
43 air only chambers. The authors state that "the exposure methods" have been described earlier

1   (Kjellstrand et al., 1980) but only reference Kjellstrand et al. (1981). In both of this and the 1981
2   study, animals were continuously exposed with only a few hours of cessation of exposure noted a
3   week for change of food and bedding. Under this paradigm, there is the possibility of additional
4   oral exposure to TCE due to grooming and consumption of TCE on food in the chamber. The
5   study was reported to be composed of two independent experiments with the exception of strain
6   NMRI which had been studied in Kjellstrand et al 1981 & 1983b. The number of animals
7   examined in this study ranged from 3-6 in each treatment group. The authors reported
8   "significant difference between the animals intended for TCE-exposure and the matched controls
9   intended for air-exposure were seen in four cases (table 1.)", and stated that the grouping effects
10   developed during the 7-day adaptation period. Premature mortality was attributed to an accident
11   for one TCE-exposed DBA male and fighting to the deaths of two TCE-exposed NZB females
12   and one B6CBA male in each air exposed chamber. Given the small number of animals
13   examined in this study in each group, such losses significantly decrease the power of the study to
14   detect TCE-induced changes. The range of initial body weights between the groups of male
15   mice for all strains was between 18 g (as mean value for the A/sn strain) and 32 g (as mean value
16   for the B6CBA strain) or ~ 44%. For females the range of initial body weights between groups
17   for all strains was 15g (as mean value for the A/sn strain) and 24 g (as mean value for the DBA
18   strain) or ~38%..
19
20   Rather than reporting % liver/body weight ratios or an extrapolated value, as was done in
21   Kjellstrand et al. (1981), this study only reported actual liver weights for treated and exposed
22   groups at the end of 30 days of exposure. The authors report final body weight changes in
23   comparison to matched control groups at the end of the exposure periods but not the changes in
24   body weight for individual animals. They report the results from statistical analyses of the
25   difference in values between TCE and air-exposed groups. A statistically significant decrease in
26   body weight was reported between TCE exposed and control mice in experiment 1 of the C57BL
27   male mice (~ 20% reduction in body weight due to TCE exposure). This group also had a slight
28   but statistically significant difference in body weight at the beginning of exposure with the
29   control group having a ~5% difference in starting weight. There was also a statistically
30   significant decrease in body weight of 20% reported after TCE exposure in one group of male
31   B6CBA mice that did not have a difference in body weight at the beginning of the experiment
32   between treatment and control groups. One group of female and both groups of male A/sn mice
33   had statistically significant decreases in body weight after TCE exposure (10% for the females,
34   and 22 and 26% decreases in the two male groups) in comparison to untreated mice of the same
35   strain. The magnitude of body weight decrease in this strain after TCE treatment also reflects
36   differences in initial body weight as there were also differences in initial body weight between
37   the two groups of both treated and untreated A/sn males that were statistically significant, 17%
38   and 10% respectively. One group of male NZB mice had a significant increase in body weight
39   after TCE exposure of 14% compared to untreated animals. A female group from the same
40   strain treated with TCE was reported to have a nonsignificant but 7% increase in final body
41   weight in comparison to its untreated group. The one group of male NMRI mice (n= 10) in this
42   study was reported to have a statistically significant 12% decrease in body weight compared to
43   controls.

1

2   For the groups of animals with reported TCE exposure-related changes in final body
3 weight compared to untreated animals, such body weight changes may also have affected the
4 liver weights changes reported. The authors do not explicitly state that they did not record liver
5 and body weights specifically for each animal, and thus would be unable to determine liver/body
6 weight ratios for each, however they do state that he animals were housed 4-6 in each cage and
7 placed in exposure chambers together. The authors only present data for body and liver weights
8 as the means for a cage group in the reporting of their results. While this approach lends more
9 certainty in their measurements than the approach taken by Kjellstrand et al (1981) as described
10 above, the relative liver/body weights cannot be determined for individual animals. It appears
11 that the authors have tried to carefully match the body weights of the control and exposed mice
12 at the beginning of the experiment to minimize the effects of initial body weight differences and
13 distinguish the effects of treatment on body weight and liver weight. However, there is no ability
14 to determine liver/body weight ratios and adjust for difference in initial body weight from
15 changes due to TCE exposure. For the groups in which there was no change in body weight after
16 TCE treatment and in which there was no difference in initial body weight between controls and
17 TCE-exposed groups, the reporting of liver weight changes due to TCE exposure is a clearer
18 reflection of TCE-induced effects and the magnitude of such effects. Nevertheless the small
19 number of animals examined in each group is still a limitation on the ability to determine the
20 magnitude of such responses and there statistical significance.
21

22   In "wild type" mice there were no reported significant differences in the initial and final
23 body weight of male or female mice before or after 30 days of TCE exposure.   For these groups
24 there was 1.76-fold and 1.80-fold of control values for liver weight in groups 1 and 2 for female
25 mice, and for males 1.84-fold and 1.62-fold of control values for groups 1 and 2, respectively.
26 For DBA mice there were no reported significant differences in the initial and final body weight
27 of male or female mice before or after 30 days of TCE exposure.   For DBA mice there was 1.87-
28 fold and 1.88-fold of control for liver weight in groups 1 and 2 for female mice, and for males
29 1.45-fold and 2.00-fold of control for groups 1 and 2, respectively.   These groups represent the
30 most accurate data for TCE-induced changes in liver weight not affected by initial differences in
31 body weight or systemic effects of TCE which resulted in decreased body weight gain. These
32 results suggest that there is more variability in TCE-induced liver weight gain between groups of
33 male than female mice.
34

35   The C57BL, B6CBA, NZB, and NMRI groups all had at least one group of male mice
36 with changes in body weight due to TCE exposure. The A/sn group not only had both male
37 groups with decreased body weight after TCE exposure (along with differences between exposed
38 and control groups at the initiation of exposure) but also a decrease in body weight in one of the
39 female groups. Thus, the results for TCE-induced liver weight change in these male groups also
40 reflect changes in body weight. These results suggest a strain-related increased sensitivity to
41 TCE toxicity as reflected by decreased body weight. For C57BL mice there was 1.65-fold and
42 1.60-fold of control for liver weight after TCE exposure was reported in groups 1 and 2 for
43 female mice, and for males 1.28-fold (the group with decreased body weight) and 1.82-fold of

1   control values for groups 1 and 2, respectively. For B6CBA mice there was 1.70-fold and 1.69-
2   fold of controls values for liver weight after TCE exposure in groups 1 and 2 for female mice,
3   and for males 1.21-fold (the group with decreased body weight) and 1.47-fold of control values
4   reported for groups 1 and 2, respectively. For the NZB mice there was 2.09-fold (n=3) and 2.08-
5   fold of control values for liver weight after TCE exposure in groups 1 and 2 for female mice and
6   for males 2.34-fold and 3.57-fold (the group with increased body weight) of control values
7   reported for groups 1 and 2, respectively. For the NMRI mice, whose results were reported for
8   one group with 10 mice, there was 1.66-fold of control value for liver weight after TCE exposure
9   for female mice and for males 1.68-fold of control value reported (a group with decreased body
10 weight). Finally, for the A/sn strain that had decreased body weight in all groups but one after
11 TCE exposure and significantly smaller body weights in the control groups before TCE exposure
12 in both male groups, the results still show TCE-related liver weight increases. For the As/n mice
13 there was 1.56-fold and 1.72-fold (a group with decreased body weight) of control value for liver
14 weight in groups 1 and 2 for female mice and for males 1.62-fold (a group with decreased body
15 weight) and 1.58-fold (a group with decreased body weight) of control values reported for
16 groups 1 and 2, respectively.
17
18   The consistency between groups of female mice of the same strain for TCE-induced liver
19 weight gain, regardless of strain examined, is striking. The largest difference within female
20 strain groups occurred in the only strain in which there was a decrease in TCE-induced body
21 weight. For males, even in strains that did not show TCE-related changes in body weight, there
22 was greater variation between groups than in females. For strains in which one group had TCE-
23 related changes in body weight and another did not, the group with the body weight decrease
24 always had a lower liver weight as well. Groups that had increased body weight after TCE
25 exposure also had an increased liver weight in comparison to the groups without a body weight
26 change. These results demonstrate the importance of carefully matching control animals to
27 treated animals and the importance of the effect of systemic toxicity, as measured by body
28 weight decreases, on the determination of the magnitude of liver weight gain induced by TCE
29 exposure. These results also show the increased variation in TCE-induced liver weight gain
30 between groups of male mice and an increase incidence of body weight changes due to TCE
31 exposure in comparison to females, regardless of strain.
32
33   In terms of strain sensitivity, it is important not only to take into account differing effects
34 on body weight changes due to TCE exposure but also to compare animals of the same age or
35 beginning weight as these parameters may also affect liver weight gain or toxicity induced by
36 TCE exposure. The authors do not state the age of the animals at the beginning of exposure and
37 report, as stated above, a range of initial body weights between the groups as much as 44 % for
38 males and 38% for females. These differences can be due to strain and age. The differences in
39 final body weight between the groups of controls, when all animals would have been 30 days
40 older and more mature, was still as much as 48% for males and 44% for females. The data for
41 female mice, in which body weight was decreased by TCE exposure only in on group in one
42 strain, suggest that the magnitude of TCE-induced liver weight increase was correlated with
43 body weight of the animals at the beginning of the experiment. For the C57BL and As/n strains,

1 female mice starting weights were averaged 17.5 g and 15.5 g, respectively, while the average
2 liver weights were 1.63-fold and 1.64-fold of control after TCE exposure, respectively. For the
3 B6CBA, "wild" type, DBA, and NZB female groups the starting body weights averaged 22.5 g,
4 21.0 g, 23.0 g, and 21.0 g, respectively, while the average liver weight increases were 1.70-fold,
5 1.78-fold, 1.88-fold, and 2.09-fold of control after TCE exposure. Thus, groups of female mice
6 with higher body weights, regardless of strain, generally had higher increases in TCE-induced
7 liver weight increases. The NMRI group of female mice, did not follow this general pattern and
8 had the highest initial body weight for the single group of 10 mice reported (i.e., 27 g) associated
9 with a 1.66-fold of control value for liver weight. It is probable that the data for these mice had
10 been collected from another study. In fact, the starting weights reported for these groups of 10
11 mice are identical to the starting weights reported for 26 mice examined in Kjellstrand et al
12 (1981). However, while this study reports a 1.66-fold of control value for liver weight after 30
13 days of TCE exposure, the extrapolated % liver/body weight given in the 1981 study for 30 days
14 of TCE exposure was 1.74-fold of control in female NMRI mice. In the Kjellstrand et al (1983b)
15 study, discussed below, 10 female mice were reported to have a 1.66-fold of control value for
16 liver weight after 30 days exposure to 150 ppm TCE with an initial starting weight of 26.7 g.
17 Thus, these data appear to be from that study. Thus, differences in study design, variation
18 between experiments, and strain differences may account for the differences results reported in
19 Kjellstrand et al (1983a) for NMRI mice and the other strains in regard to the relationship to
20 initial body weight and TCE response of liver weight gain.
21
22   These data suggest that initial body weight is a factor in the magnitude of TCE-induced
23 liver weight induction rather than just strain. For male mice, there appeared to be a difference
24 between strains in TCE-induced body weight reduction, which in turn affects liver weight. The
25 DBA and "wild" type mice appeared to be the most resistant to this effect (with no groups
26 affected), while the C57BL, B6CBA, and NZB strains appearing to have at least one group
27 affected, and the A/sn strain having both groups of males affected. Only one group of NMRI
28 mice were reported in this study and that group had TCE-induced decreases in body weight. As
29 stated above there appeared to be much greater differences between groups of males within the
30 same strain in regard to liver weight increases than for females and that the increases appeared to
31 be affected by concurrent body weight changes. In general the strains and groups within strain,
32 that had TCE-induced body weight decreases, had the smallest increases in liver weight, while
33 those with no TCE-induced changes in body weight in comparison to untreated animals (i.e.,
34 "wild type" and DBA) or had an actual increase in body weight (one group of NZB mice) had
35 the greatest TCE-induced increase in liver weight. Therefore, only examining liver weight in
36 males rather than % liver/ body weight ratios would not be an accurate predictor of strain
37 sensitivity at this dose due to differences in initial body weight and TCE-induced body weight
38 changes.
39
40 **2.2.6. Kjellstrand et al. 1983(b)**
41
42   This study was conducted in male and female NMRI mice with a similar design as
43 Kjellstrand et al. (1983a). The ages of the mice were not given by the authors. Animals were

1   housed 10 animals per cage and exposed from 30 to 120 days at concentrations ranging from 37
2   to 3600 ppm TCE. TCE was stabilized with 0.01% thymol and 0.03% diisopropylene.   Animals
3   were exposed continuously with exposure chambers being opened twice a week for change of
4   bedding food and water resulting in a drop in TCE concentration of ~ 1 hour. A group of mice
5   was exposed intermittently with TCE at night for 16 hours. This paradigm results not only in
6   inhalation exposure but also oral exposure from TCE adsorption to food and grooming
7   behaviour. The authors state that "the different methodological aspects linked to statistical
8   treatment of body and organ weights have been discussed earlier (Kjellstrand et al., 1981). The
9   same air-exposed control was used in three cases." The design of the experiment, in terms of
10   measurement of individual organ and body weights and the inability to assign a % liver/body
11   weight for each animal, and limitations are similar to that of Kjellstrand et al. (1983b). The
12   exposure design was for groups of male and female mice to be exposed to 37 ppm, 75 ppm, 150
13   ppm, and 300 ppm TCE continuously for 30 days (n = 10 per gender and group except for the 37
14   ppm exposure groups) and then for liver weight and body weight to be determined. Additional
15   groups of animals were exposed for 150 ppm continuously for 120 days (n = 10). Intermittent
16   exposure of 4 hours/day for 7 days a week were conducted for 120 days at 900 ppm and
17   examined immediately or 30 days after cessation of exposure (n = 10). Intermittent exposures of
18   16 hours/day at 255 ppm group (n = 10), 8 hours/day at 450 ppm, 4 hours/day at 900 ppm, 2
19   hours/day at 1800 ppm, and 1 hour/day at 3600 ppm 7 days/week for 30 days were also
20   conducted (n = 10 per group).
21
22   As in Kjellstrand et al. (1983a), body weights for individual animals were not recorded in
23   a way that the initial and final body weights could be compared. The approach taken by the
24   authors was to match the control group at the initiation of exposure and compare control and
25   treated average values. At the beginning of the experiment only one group began the experiment
26   with a statistically significant change in body weight between treated and control animals
27   (female mice exposed 16 hours a day for 30 days).   In regard to final body weight, which would
28   indicate systemic TCE toxicity, 5 groups had significantly decreased body weight (i.e., males
29   exposed to 150 ppm continuously for 30 or 120 days, males and females exposed continuously to
30   300 ppm for 30 days) and 2 groups significantly increased body weight (i.e., males exposed to
31   1800 ppm for 2 hours/day and 3600 ppm for 1 hour/day for 30 days) after TCE exposure. Thus,
32   the accuracy of determining the effect of TCE on liver weight changes, reported by the authors in
33   this study for groups in which body weight were also affected by TCE exposure, would be
34   affected by similar issues as for data presented by Kjellstand et al. (1983a). In addition,
35   comparison in results between the 37 ppm exposure groups and those of the other groups would
36   be affected by difference in number of animals examined (10 vs. 20). As with Kjellstrand et al
37   (1983 a), the ages of the animals in this study are not given by the author. Difference in initial
38   body weight (which can be affected by age and strain) reported by Kjellstrand et al (1983a)
39   appeared to be correlated with the degree of TCE-induced change in liver weight. Although each
40   exposed group was matched to a control group with a similar average weight, the average initial
41   body weights in this study varied between groups (i.e., as much as 14% in female control, 16%
42   in TCE-exposed female mice, 12% in male control, and 16% in male exposed mice).
43

1   For female mice exposed from 37 ppm to 300 ppm TCE continuously for 30 days, only
2   the 300 pm group experienced a 16% decrease in body weight between control and exposed
3   animals. Thus, liver weight increased reported by this study after TCE exposure were not
4   affected by changes in body weight for exposures below 300 ppm in female mice. Initial body
5   weights in the TCE-exposed female mice were similar in each of these groups (i.e., range of 29.2
6   – 31.6 g, or 8%), with the exception of the females exposed to 150 ppm TCE for 30 days (i.e.,
7   initial body weight of 27.3 g), reducing the effects of differences in initial body weight on TCE-
8   induced liver weight induction. Exposure to TCE continuously for 30 days resulted in a dose-
9   dependent change in liver weight in female mice with 1.06-fold, 1.27-fold, 1.66-fold, and 2.14-
10   fold of control values reported for liver weight at 37 ppm, 75 ppm, 150 ppm, and 300 ppm TCE,
11   respectively. The increase at 300 ppm in females was accompanied by statistically significant
12   decreased body weight in the TCE exposed groups compared to control (~ 16%). Thus, the
13   response in liver weight gain at that exposure is in the presence of toxicity. However, the TCE-
14   induced increases in liver weight consistently increased with dose of TCE in a linear fashion.
15
16   For male mice exposed to 37 to 300 ppm TCE continuously for 30 days, both the 150
17   ppm and 300 ppm exposed groups experienced a 10% and 18% decrease in body weight after
18   TCE exposure, respectively. The 37 ppm and 75 ppm groups did not have decreased body
19   weight due to TCE exposure but varied by 12% in initial body weight. Thus, there are more
20   factors affecting reported liver weight increases from TCE exposure in the male than female
21   mice, most importantly toxicity. Exposure to TCE continuously for 30 days resulted in liver
22   weights of 1.15-fold, 1.50-fold, 1.69-fold, and 1.90-fold of control for 37 ppm, 75 ppm, 150
23   ppm, and 300 ppm, respectively. The flattening of the dose-response curve for liver weight in
24   the male mice is consistent with the effects of toxicity at the two highest doses and thus the
25   magnitude of response at these doses should be viewed with caution. Consistent with Kjellstrand
26   et al. (1983a) results, male mice in this study appeared to have a higher incidence of TCE-
27   induced body weight changes than female mice.
28
29   The effects of extended exposure, lower durations of exposure but at higher
30   concentrations, and of cessation of exposure were examined for 150 ppm and higher doses of
31   TCE. Mice exposed to TCE at 150 ppm continuously for 120 days were reported to have
32   increased liver weight (i.e., 1.57-fold of control for females and 1.49-fold of control for males),
33   but in the case of male mice, also to have a significant decrease in body weight of 17% in
34   comparison to control groups. Increasing the exposure concentration to 900 ppm TCE and
35   reducing exposure time to 4 hours/day for 120 days also resulted in increased liver weight (i.e.,
36   1.35-fold of control for females and 1.49-fold of controls for males) but with a significant
37   decrease in body weight in females of 7% in comparison to control groups. For mice that were
38   exposed to 150 ppm TCE for 30 days and then examined 120 days after the cessation of
39   exposure, liver weights were 1.09-fold of control for female mice and the same as controls for
40   male mice. With the exception of 1800 ppm and 3600 ppm TCE groups exposed at 2 and 1 hour,
41   respectively, exposure from 225 ppm, 450 and 900 ppm at 16 hours, 8 hours, and 4 hours,
42   respectively for 30 days did not result in decreased body weight in males or female mice. These
43   exposures did result in increased liver weights in relation to control groups and for female mice

1   the magnitude of increase was similar (i.e., 1.50-fold, 1.54-fold, and 1.51-fold of control for liver
2   weight after exposure to 225 ppm TCE 16 hours/day, 450 ppm TCE 8 hours/day, and 900 ppm
3   TCE 4 hours/day, respectively). For these groups, initial body weights varied by 13% in females
4   and 14% in males. Thus, under circumstances without body weight changes due to TCE toxicity,
5   liver weight appeared to have a consistent relationship with the product of duration and
6   concentration of exposure in female mice. For male mice, the increases in TCE-induced liver
7   weight were more variable (i.e., 1.94-fold, 1.74-fold, and 1.61-fold of control for liver weight
8   after exposure to 225 ppm TCE 16 hours/day, 450 ppm TCE 8 hours/day, and 900 ppm TCE 4
9   hours/day, respectively) with the product of exposure duration and concentration did not result in
10 a consistent response in males (e.g., a lower dose for a longer duration of exposure resulted in a
11 greater response than a larger dose at a shorter duration of exposure).
12
13   Kjellstrand et al. (1983b) reported light microscopic findings from this study and report
14 that "after 150 ppm exposure for 30 days, the normal trabecular arrangement of the liver cells
15 remained. However, the liver cells were generally larger and often displayed a fine vacuolization
16 of the cytoplasm. The nucleoli varied slightly to moderately in size and shape and had a finer,
17 granular chromatin with a varying basophilic staining intensity. The Kupffer cells of the
18 sinusoid were increased in cellular and nuclear size. The intralobular connective tissue was
19 infiltrated by inflammatory cells. There was not sign of bile stasis. Exposure to TCE in higher
20 or lower concentrations during the 30 days produced a similar morphologic picture. After
21 intermittent exposure for 30 days to a time weighted average concentration of 150 ppm or
22 continuous exposure for 120 days, the trabecular cellular arrangement was less well preserved.
23 The cells had increased in size and the variations in size and shape of the cells were much
24 greater. The nuclei also displayed a greater variation in basophilic staining intensity, and often
25 had one or two enlarged nucleoli. Mitosis were also more frequent in the groups exposed for
26 longer intervals. The vacuolization of the cytoplasm was also much more pronounced.
27 Inflammatory cell infiltration in the interlobular connective tissue was more prominent. After
28 exposure to 150 ppm for 30 days, followed by 120 days of rehabilitation, the morphological
29 picture was similar to that of the air-exposure controls except for changes in cellular and nuclear
30 sizes."   Although not reporting comparisons between changes in male and female mice in the
31 results section of the paper, the authors state in the discussion section that "However, liver mass
32 increase and the changes in liver cell morphology were similar in TCE-exposed male and female
33 mice."
34
35   The authors do not present any quantitative data on the lesions they describe, especially
36 in terms of dose-response. Most of the qualitative description is for the 150 ppm exposure level,
37 in which there are consistent reports of TCE induced body weight decreases in male mice. The
38 authors suggest that lower concentrations of TCE give a similar pathology as those at the 150
39 ppm level, but do not present data to support that conclusion. Although stating that Kupffer cells
40 were increased in cellular and nuclear size, no differential staining was applied light microscopy
41 sections distinguish Kupffer from endothelial cells lining the hepatic sinusoid in this study.
42 Without differential staining such a determination is difficult at the light microscopic level.
43 Indeed, Goel et al. (1992) describe proliferation of sinusoidal endothelial cells after 1000

1   mg/kg/day and 2000 mg/kg/day TCE exposure for 28 days in male Swiss mice. However, the
2   described inflammatory cell infiltrates in the Kjellstrand et al. (1983b) study are consistent with
3   invasion of macrophages and well as polymorphonuclear cells into the liver, which could
4   activate resident Kupffer cells. Although not specifically describing the changes as consistent
5   with increased polyploidization of hepatocytes, the changes in cell size and especially the
6   continued change in cell size and nuclear staining characteristics after 120 days of cessation of
7   exposure are consistent with changes in polyploidization induced by TCE. Of note is that in the
8   histological description provided by the authors, although vacuolization is reported and
9   consistent with hepatotoxicity or lipid accumulation which is lost during routine histological
10   slide preparation, there is no mention of focal necrosis or apoptosis resulting from these
11   exposures to TCE.

12
13   **2.2.7.   Buben and O'Flaherty 1985**
14
15   This study was conducted with older mice than those generally used in chronic exposure
16   assays (Male Swiss-Cox outbred mice between 3 and 5 months of age) with a weight range
17   reported between 34 to 45 g. The mice were administered distilled TCE in corn oil by gavage 5
18   times a week for 6 weeks at exposure concentrations of either 0, 100, 200, 400, 800, 1600, 2400,
19   or 3200 mg TCE /kg/day. While 12-15 mice were used in most exposure groups, the 100- and
20   3200- mg/kg groups contained 4-6 mice and the two control groups consisted of 24 and 26 mice.
21   Liver toxicity was determined by "liver weight increases, decreases in liver glucose-6-phosphate
22   (G6P) activity, increases in liver triglycerides, and increases in serum glutamate-pyruvate
23   transaminase (SGPT) activity". Livers were perfused with cold saline prior to testing for weight
24   and enzyme activity and hepatic DNA was measured.

25
26   The authors reported the mice to tolerate the 6-week exposed with TCE with few deaths
27   occurring except at the highest dose and that such deaths were related to central nervous system
28   depression. Mice in all dose groups were reported to continue to gain weight throughout the 6-
29   week dosing period. However, TCE exposure caused "dose-related increases in liver weight to
30   body weight ratio and since body weight of mice were generally unaffected by treatment, the
31   increases represent true liver weight increases." Exposure concentrations, as low as 100
32   mg/kg/day, were reported to be "sufficient to cause statistically significant increase in the liver
33   weight/body weight ratio," and the increases in liver size to be "attributable to hypertrophy of the
34   liver cells, as revealed by histological examination and by a decrease in the DNA concentration
35   in the livers." Mice in the highest dose group were reported to display liver weight/body weight
36   ratios that were about ~75% greater than those of controls and even at the lowest dose there was
37   a statistically significant increase (i.e., control liver/ body weight % was reported to be 5.22 ±
38   0.09 % vs. 5.85 ± 0.20 % in 100 mg/kg/day exposed mice). The % liver/body ratios were 5.22 ±
39   0.09, 5.84 ± 0.20, 5.99 ± 0.13, 6.51 ± 0.12, 7.12 ± 0.12, 8.51 ± 0.20, 8.82 ± 0.15 and 9.12 ± 0.15
40   % for control (n= 24), 100 (n=5), 200 (n=12), 400 (n=12), 800 (n= 12), 1600 (n=12), 2400 (n=
41   12) and 3200 (n= 4) mg/kg/day TCE. This represents 1.12-fold, 1.15-fold, 1.25-fold, 1.36-fold,
42   1.63-fold, 1.69-fold, and 1.75-fold of control for these doses. All dose groups of TCE induced a
43   statistically significant increase in liver/body weight ratios. For the 200 through 1600 mg/kg

1   exposure levels, the magnitudes of the increases in TCE exposure concentrations were similar to
2   the magnitudes of TCE-induced increases in % liver/body weight ratios (i.e., a ~ 2-fold increase
3   in TCE dose resulted in ~ 1.7 fold increase change in % liver/body weight).
4
5   TCE exposure was reported to induce a dose-related trend towards increased
6   triglycerides (i.e., control values of 3.08 ± 0.29 vs. 6.89 ± 1.40 at 2400 mg/kg TCE) with
7   variation of response increased with TCE exposure. For liver triglycerides the reported values in
8   mg/g liver were 3.08 ± 0.29 (n= 24), 3.12 ± 0.49 (n= 5), 4.41 ± 0.76 (n= 12), 4.53 ± 1.05 (n=12),
9   5.76 ± 0.85 (n=12), 5.82 ± 0.93 (n=12), 6.89 ± 1.40 (n= 12), and 7.02 ± 0.69 (n= 4) for control,
10   100, 200, 400, 800, 1600, 2400, and 3200 mg/kg/day dose groups, respectively.
11
12   For G6P the values in μg phosphate/mg protein/20 minutes were 125.5 ± 3.2 (n=12),
13   117.8 ± 6.0 (n=5), 116.4 ± 2.8 (n=9), 117.3 ± 4.6 (n=9), 111.7 ± 3.3. (n=9), 89.9 ± 1.7 (n= 9),
14   83.8 ± 2.1 (n=8), and 83.0 ± 7.0 (n=3) for the same dose groups. Only the 2400 mg/kg/day
15   dosing group was reported to be statistically significantly increased for triglycerides after TCE
16   exposure although there appeared to be a dose-response. For decreases in G6P the 800
17   mg/kg/day and above doses were statistically significant. The numbers of animals varied
18   between groups in this study but in particular only a subset of the animals were tested for G6P
19   with the authors providing no rationale for the selection of animals for this assay. The
20   differences in the number of animals per group and small number of animals per group affected
21   the ability to determine a statistically significant change in these parameters but the changes in
22   liver weights were robust enough and variation small enough between groups that all TCE-
23   induced changes were described as statistically significant. The livers of TCE treated mice,
24   although enlarged, were reported to appear normal. A dose-related decrease in glucose-6-
25   phophatase activity was reported with similar small decreases (~ 10%) observed in the TCE
26   exposed groups that did not reach statistical significance until the dose reached 800 mg/kg TCE
27   exposure. SGPT activity was not observed to be increased in TCE-treated mice except at the two
28   highest doses and even at the 2400 mg/kg dose half of the mice had normal values. The large
29   variability in SGPT activity was indicative of heterogeneity of this response between mice at the
30   higher exposure levels for this indicator of liver toxicity. However, the results of this study also
31   demonstrate that hepatomegaly was a robust response that was observed at the lowest dose
32   tested, was dose-related, and was not accompanied by toxicity.
33
34   Liver histopathology and DNA content were determined only in control, 400, and 1600
35   mg/kg TCE exposure groups. DNA content was reported to be significantly decreased from 2.83
36   ± 0.17 mg/g liver in controls to 2.57 ± 0.14 in 400 mg/kg TCE treated group, and to 2.15 ± 0.08
37   mg/kg liver in the 1600 mg/kg exposed group. This result was consistent with a decreased
38   number of nuclei per gram of liver and hepatocellular hypertrophy. Liver degeneration was
39   reported as swollen hepatocytes and to be common with treatment. "Cells had indistinct borders;
40   their cytoplasm was clumped and a vesicular pattern was apparent. The swelling was not simply
41   due to edema, as wet weight/dry weight ratios did not increase." Karyorhexis (the disintegration
42   of the nucleus) was reported to be present in nearly all specimens and suggestive of impending
43   cell death. A qualitative scale of negative, 1, 2, 3, or 4 was given by the authors to rate their

findings without further definition or criterion given for the ratings. "No Karyorhexis, necrosis, or polyploidy was reported in controls, but a score of 1 for Karyorhexis was given for 400 mg/kg TCE and 2 for 1600 mg/kg TCE." Central lobular necrosis reported to be present only at the 1600 mg/kg TCE exposure level and as a score of 1. "Polyploidy was also characteristic in the central lobular region" with a score of 1 for both 400 mg/kg and 1600 mg/kg TCE. The authors reported that "hepatic cells had two or more nuclei or had enlarged nuclei containing increased amounts of chromatin, suggesting that a regenerative process was ongoing" and that there were no fine lipid droplets in TCE exposed animals. The finding of "no polyploidy" in control mouse liver is unexpected given that binucleate and polyploid hepatocytes are a common finding in the mature mouse liver. It is possible that the authors were referring to unusually high instances of "polyploidy" in comparison to what would be expected for the mature mouse. The score given by the authors for polyploidy did not indicate a difference between the two TCE exposure treatments and that it was of the lowest level of severity or occurrence. No score was given for centrolobular hypertrophy although the DNA content and liver weight changes suggested a dose response. The "Karyorhexis" described in this study could have been a sign of cell death associated with increased liver cell number or dying of maturing hepatocytes associated with the increased ploidy, and suggests that TCE treatment was inducing polyploidization. Consistent with enzyme analyses, centrilobular necrosis was only seen at the highest dose and with the lowest qualitative score, indicating that even at the highest dose there was little toxicity.

Thus, the results of this study of TCE exposure for 6 weeks, is consistent with acute studies and show that the region of the liver affected by TCE is the centralobular region, that hepatocellular hypertrophy is observed in that region, and that increased liver weight is induced at the lowest exposure level tested and much lower than those inducing overt toxicity. These authors suggest polyploidization is occurring as a result of TCE exposure although a quantitative dose-response cannot be determined from these data.

2.2.8. Channel et al. 1998

This study was performed in male hybrid B6C3F1/CrlBR mice (13 weeks-old, 25-30 grams) and focused on indicators of oxidative stress. TCE was administered by oral gavage 5 days a week in corn oil for up to 55 days for some groups. Although the study design indicated that water controls, corn oil controls, and exposure levels of 400, 800, and 1200 mg/kg day TCE in corn oil, results were not presented for water controls for some parameters measured. Initial body weights and those recorded during the course of the study were not reported for individual treatment groups. Liver samples were collected on study days 2, 3, 6, 10, 14, 21, 28, 35, 42, 49, and 56. Histopathology was studied from a single section taken from the median lobe. Thiorbarbiturate acid-reactive substances (TBARS) were determined from whole liver homogenates. Nuclei were isolated from whole liver homogenates and DNA assayed for 8-hydroxydeoxyguanosine (8-OHdG). There was no indication that parenchymal cell and nonparenchymal cells were distinguished in the assay. Free radical electron paramagnetic resonance (EPR) for total radicals was analyzed in whole liver homogenates. For peroxisome detection and analysis, livers from 3 mice from the 1200 mg/kg TCE and control (oil and water)

1 groups were analyzed via electron microscopy. Only centrilobular regions, the area stated by the
2 authors to be the primary site of peroxisome proliferation, were examined. For each animal, 7
3 micrographs of randomly chosen hepatocytes immediately adjacent to the central vein were
4 examined with peroxisomal area to cytoplasmic area, the number of peroxisomes per unit area of
5 cytoplasm, and average peroxisomal size quantified. Proliferation cell nuclear antigen (PCNA),
6 described as a marker of cell cycle except G0, was examined in histological sections for a
7 minimum of 18 fields per liver section. The authors did not indicate what areas of the liver
8 lobule were examined for PCNA. Apoptosis was detected on liver sections using a apoptosis kit
9 using a single liver section from the median lobe and based on the number of positively labeled
10 cells per 10 mm^2 in combination with the morphological criteria for apoptosis of Columbano et
11 al. (1985). However, the authors did not indicate what areas of the liver lobule were specifically
12 examined.
13
14   The authors reported that body weight gain was not adversely affected by TCE dosing of
15 the time course of the study but did not show the data. No gross lesions were reported to be
16 observed in any group. For TBARS no water control data was reported by the authors. Data was
17 presented for 6 animals per group for the corn oil control group and the 1200 mg/kg group (error
18 bars representing the SE). No data was presented without corn oil so that the effects of corn oil
19 on the first day of the study (day 2 of dosing) could not be determined. After 2 and 3 days of
20 dosing the corn oil and 1200 mg/kg TCE groups appeared to have similar levels of TBAR
21 detected in whole liver as nmol TBARS/mg protein. However, by day 6 the corn oil treated
22 control had a decrease in TBAR that continued until day 15 where the level was ~ 50% of that
23 reported on days 2 and 3. The variation between animals as measured by SE was reported to be
24 large on day 10. By day 20 there was a slight increase in variation that declined by day 35 and
25 stayed the same through day 55. For the TCE exposed group the TBARs remained relatively
26 consistent and began to decline by about day 20 to a level that similar to the corn oil declines by
27 day 35. Therefore, corn oil alone had a significant effect on TBAR detection inducing a decline
28 by 6 days of administration that persisted thought 55 days. TCE administration at the 1200
29 mg/kg dose in corn oil appeared to have a delayed decline in TBARS. The authors interpreted
30 this pattern to show that lipid peroxidation was elevated in the 1200 mg/kg TCE group at day 6
31 over corn oil. However, corn oil alone induced a decrease in TBARs. At no time was TBARS in
32 TCE treatment groups reported to be greater than the initial levels at days 2 and 3, a time in
33 which TCE and corn oil treatment groups had similar levels. Rather than inducing increasing
34 TBARS over the time course of the study TCE, at the 1200 mg/kg dose, appeared to delay the
35 corn oil induced suppression of TBARs detection. Because the authors did not present data for
36 aqueous control animals, the time course of TBARS detection in the absence of corn oil, cannot
37 be established.
38
39   For the 800 and 400 mg/kg TCE data the authors presented a figure, without standard
40 error information, for up to 35 days that shows little difference between 400 mg/kg TCE
41 treatment and corn oil suppression of TBAR induction. There was little difference between the
42 patterns of TBAR detection for 800 and 400 mg/kg TCE, indicating that both delayed TBAR
43 suppression by corn oil to a similar extent and did not induce greater TBAR than corn oil alone.

1

2   For 8-OHdG levels, the authors report that elevations were modest with the greatest
3   increase noted in the 1200 mg/kg day TCE treatment group of 196% of oil controls on day 56.
4   Levels fluctuated throughout the study with most of the time points that were elevated showing
5   129% of control for the 1200 mg/kg/day group. Statistically significant elevations were noted on
6   days 2, 10, 28, 49 and 56 with depression on day 3. On all other days (i.e., days 6, 14, 21, 35 and
7   42) the 8-OHdG values were similar to those of corn oil controls. No statistically significant
8   effects were reported to be observed at lower doses. The figure presented by the authors shows
9   the % of controls by TCE treatment at 1200 mg/kg/day but not the control values themselves.
10   The pattern by corn oil is not shown and neither is the standard error of the data. As a percent of
11   control values the variations were very large for many of the data points and largest for the data
12   given at day 55 in which the authors report the largest difference between control and TCE
13   treatment. There was no apparent pattern of elevation in 8-OHdG when the data was presented
14   in this manner. Because the data for the corn oil control was not given, as well as no data given
15   for aqueous controls, the effects of corn oil alone cannot be discerned.

16

17   Given that for TBARS corn oil had a significant effect and showed a pattern of decline
18   after 6 days, with TCE showing a delayed decline, it is especially important to discern the effects
19   of corn oil and to see the pattern of the data. At time points when TBARS levels were reported
20   to be the same between corn oil and TCE (days 42, 49 and 56) the pattern of 8-OHdG was quite
21   different with a lower level at day 42 a slightly increased level at day 49 and the highest
22   difference reported at day 56 between corn oil control and TCE treated animals. The authors
23   report that the pattern of "lipid peroxidation" to be similar between the 1200 and 800 mg/kg
24   doses of TCE but for there to be no significant difference between 800 mg/kg TCE and corn oil
25   controls. Thus the pattern of TBARS as a measure of lipid peroxidation and 8-OHdG level in
26   nuclear DNA did not match.

27

28   In regard to total free radical levels as measured by EPR, results were reported for the
29   1200 mg/kg TCE as a signal that was subtracted from control values with the authors stating that
30   only this dose level induced an elevation significantly different from controls. Again, aqueous
31   control values were not presented to discern the effects of corn oil or the pattern that may have
32   arisen with time of corn oil administration. The pattern of total free radical level appeared to
33   differ from that of lipid peroxidation and for that of 8-OHdG DNA levels with no changes at
34   days 2, 3, a peak level at day 6, a rapid drop at day 10, mild elevation at day 20, and significant
35   decrease at day 49. The percentage differences between control and treated values reported at
36   day 6 and 20 by the authors was not proportional to the fold-difference in signal indicating that
37   there was not a consistent level for control values over the time course of the experiment. While
38   differences in lipid peroxidation detection between 1200 mg/kg TCE and corn oil control were
39   greatest at day 14, total free radicals showed their biggest change between corn oil controls and
40   TCE exposure on day 6, time points in which 8-OHdG levels were similar between TCE
41   treatment and corn oil controls. Again, there was no reported difference between corn oil control
42   and the 800 mg/kg TCE exposed group in total free radical formation but for lipid peroxidation
43   the 800 mg/kg TCE exposed group had a similar pattern as that of 1200 mg/kg TCE.

1   Only the 1200 mg/kg group was evaluated for peroxisomal proliferation at days 6, 10,
2   and 14. Thus, correlations with peroxisome proliferation and other parameters in the report at
3   differing times and TCE exposure concentrations could not be made. The authors report that
4   there was a treatment and time effect for percent peroxisomal area, a "treatment only "effect for
5   number of peroxisome and no effect for peroxisomal size. They also report that hepatocytes
6   examined from corn oil control rats were no different that those from water control rats for all
7   peroxisomal parameter, thus discounting a vehicle effect. However, there was an effect on
8   peroxisomal size between corn oil control and water with corn oil decreasing the peroxisomal
9   size in comparison to water on all days tested. The highest TCE-induced percent peroxisomal
10   area and number occurred on day 10 of the 3 time points measured for this dose and the fold
11   increase was ~ 4.5 fold and ~3.1 fold increase, respectively. The day-10 peak in peroxisomal
12   area and number does not correlate with the reported pattern of free radical or 8-OHdG
13   generation.
14

15   For cell proliferation and apoptosis, data were given for days 2, 6, 10, 14, and 21 in a
16   figure. PCNA cells, a measure of cells that have undergone DNA synthesis, was elevated only
17   on day 10 and only in the 1200 mg/kg/day TCE exposed group with a mean of ~ 60 positive
18   nuclei per 1000 nuclei for 6 mice (~ 6%). Given that there was little difference in PCNA
19   positive cells at the other TCE doses or time points studied, the small number of affected cells in
20   the liver could not account for the increase in liver size reported in other experimental paradigms
21   at these doses. The PCNA positive cells as well as "mitotic figures" were reported to be present
22   in centrilobular, midzonal, and periportal regions with no observed predilection for a particular
23   lobular distribution. No data was shown regarding any quantitative estimates of mitotic figures
24   and whether they correlated with PCNA results. Thus, whether the DNA synthesis phases of the
25   cell cycle indicated by PCNA staining were indentifying polyploidization or increased cell
26   number cannot be determined. The authors reported that there was no cytotoxicity manifested as
27   hepatocellular necrosis in any dose group and that there was no significant difference in
28   apoptosis between treatment and control groups with data not shown. The extent of apoptosis in
29   any of the treatment groups, or which groups and timepoints were studied for this effect cannot
30   be determined. No liver weight or body weight data was provided in this study.
31

32   These results confirm that as a vehicle corn oil is not neutral in its affects in the liver. The
33   TBARS results indicate a reduction in detection of TBARS in the liver with increasing time of
34   exposure to corn oil alone. Although control animals "treated with water" gavage were studied,
35   only the results for peroxisome proliferation were presented by the study so that the effects of
36   corn oil gavage were not easy to discern. In addition, the data was presented in such a way for 8-
37   OHdG and total free radical changes that the pattern of corn oil administration was obscured. It
38   is not apparent from this study that TCE exposure induces oxidative damage.
39

40   **2.2.9. Dorfmueller et al. 1979**
41

42   The focus of this study was the evaluation of "teratogenicity and behavioral toxicity with
43   inhalation exposure of maternal rats" to TCE. Female Long-Evans hooded rats (n=12) of ~ 210

1   g weight were treated with 1800 ± 200 ppm TCE for 6 hours/day 5 days/week for 22 ± 6 days
2   (until pregnancy confirmation) continuing through day 20 of gestation. Control animals were
3   exposed 22 ± 3 days before pregnancy confirmation. The TCE used in this study contained 0.2%
4   epichlorhydrin. Body weights were monitored as well as maternal liver weight at the end of
5   exposure. Other than organ weight, no other observations regarding the liver were reported in
6   this study. The initial weights of the dams were 212 ± 39 g (mean $\pm$ SD) and 204 ± 35 g for
7   treated and control groups, respectively. The final weights were 362 ± 32 g and 337 ± 48 g for
8   treated and control groups, respectively. There was no indication of maternal toxicity by body
9   weight determinations as a result of TCE exposure in this experiment and there was also no
10 significant difference in absolute or relative % liver/body weight between control and treated
11 female rats in this study.
12
13 **2.2.10. Kumar et al. 2001**
14
15   In this study, adult male Wistar rats (130 ± 10 g body weight) were exposed to $376 \pm$
16 1.76 ppm TCE ("AnalaR grade") for 8 , 12, and 24 weeks for 4 hours/day 5 days/week. The
17 ages of the rats were not given by the authors. Each group contained 6 rats. The animals were
18 exposed in whole body chambers and thus additional oral exposure was probable. Along with
19 histopathology of light microscopic sections, enzymatic activities of alkaline phosphatase and
20 acid phosphatase, glutamic oxoacetate transaminase, glutamic pyruvate transaminase, reduced
21 glutathione and "total sulphydryl" were assayed in whole liver homogenates as well as total
22 protein. The authors state that "the size and weight of the liver were significantly increased after
23 8, 12, and 24 weeks of TCE exposure." However, the authors do not report the final body weight
24 of the rats after treatment nor do they give quantitative data of liver weight changes. In regard to
25 histopathology, the authors state "After 8 weeks of exposure enlarged hepatocytes, with uniform
26 presence of fat vacuoles were found in all of the hepatocytes affecting the periportal, midzonal,
27 and centrilobular areas, and fat vacuoles pushing the pyknosed nuclei to one side of hepatocytes.
28 Moreover congestion was not significant. After exposure of 12 and 24 weeks, the fatty changes
29 became more progressive with marked necrosis, uniformly distributed in the entire organ." No
30 other description of pathology was provided in this report. In regard to the description of fatty
31 change, the authors only do conventional H&E staining of sections with no precautions to
32 preserve or stain lipids in their sections. The authors provide a table with histological scoring of
33 simply + or – for minimal, mild or moderate effects and do not define the criteria for that
34 scoring. There is also no quantitative information given as to the extent, nature, or location of
35 hepatocellular necrosis. The authors report "no change was observed in GOT and GPT levels of
36 liver in all the three groups. The GSH level was significantly decreased while TSH level was
37 significantly increased during 8, 12, and 24 weeks of TCE exposure. The acid and alkaline
38 phosphatases were significantly increased during 8, 12, and 24 weeks of TCE exposure." The
39 authors present a series of figures that are poor in quality to demonstrate histopathological TCE-
40 induced changes. No mortality was observed from TCE exposure in any group despite the
41 presence of liver necrosis.

1 **2.2.11. Kawamoto et al. 1988**
2
3   The focus of this study was the long-term effects of TCE treatment on induction of
4 metabolic enzymes in male adult Wistar rats. The authors reported that 8 rats weighing 200 g
5 were treated with 2.0 g/kg TCE in olive oil administered subcutaneously twice a week for 15
6 weeks with 7 rats serving as olive oil controls. In a separate experiment, 5 rats were injected
7 with 1.0 g/kg TCE in olive oil i.p. once a day for 5 continuous days. For comparative purposes
8 groups of 5 rats each were administered 3-methylcholanthrene (20 mg/kg in olive oil i.p),
9 Phenobarbital (80 mg/kg in saline i.p) for 4 days as well as ethanol administered in drinking
10 water containing 10% ethanol for 14 days. Microsomes were prepared one week after the last
11 exposure from rats administered TCE for 15 weeks and 24 hours after the last exposure for the
12 other treatments.
13
14   Body weights were reported to be slightly less for the TCE treated group than for controls
15 with the initial weights, shown in a figure, to be similar for the first weeks of exposure. At 15
16 weeks there appeared to be ~ 7.5% difference in mean body weights between control and TCE
17 treated rats which the authors reported to not be significantly different. Organ weights at the
18 termination of the experiment were reported to only be different for the liver with a 1.21-fold of
19 control value reported as a percentage of body weight with TCE treatment. The authors report
20 their increase in liver weights in male rats from subcutaneous exposure to TCE in olive oil (2.0
21 g/ kg) to be consistent with the range of liver weight gain in rats reported by Kjellstrand et al
22 (1981) for 150 ppm TCE inhalation exposure (see comments on that study above). The 5-day
23 i.p. treatment with TCE was also reported to only produce increased liver weight but the data
24 was not shown and the magnitude of the percentage increase was not given by the authors. No
25 liver pathology results were studied or reported as well.
26
27   Along with an increase in liver weight, 15-week treatment with TCE was reported to
28 cause a significant increase of microsomal protein/g liver of ~20% (10.64 ± 0.88 vs. 12.58 ±
29 0.71 mg/g liver for olive oil controls and TCE treatment, respectively). Microsomal cytochrome
30 P450 content was reported to show a mild increase that was not statistically significant of 1.08-
31 fold (1.342 ± 0.205 vs. 1.456 ± 0.159 nmol/mg protein for olive oil controls and TCE treatment,
32 respectively) of control. However, Cytochrome P-450 content showed 1.28-fold of control value
33 (14.28 ± 2.41 vs. 18.34 ± 2.31 nmol/g liver for olive oil controls and TCE treatment,
34 respectively) in terms of g/liver. Chronic treatment of TCE was also reported to cause a
35 significant increase in cytochrome b-5 level (~1.35-fold of control) and NADPH-cytochrome c
36 reductase activity (~ 1.50-fold of control) in g/liver.
37
38   The 5-day TCE treatment via the i.p. route of administration was reported to cause a
39 significant increase in microsomal protein (~ 20%), induce cytochrome P450 (~ 50% increase
40 g/liver and 22% increase in microsomal protein), but to also increase cytochrome b-5 and
41 NADPH-cytochrome c reductase activity by 50% and 70% in g/liver, respectively. Although
42 weaker, 5-day i.p treatment with TCE induced an enzyme pattern more similar to that of
43 Phenobarbital and ethanol rather methylcholanthrene (i.e., increased cytochrome P-450 but not

1 microsomal protein and NADPH-cytochrome c reductase). Direct quantitative comparisons of
2 vehicle effects and potential impact on response to TCE treatments for 15 weeks subcutaneous
3 exposure and 5-day i.p. exposure could not be made as baseline levels of all enzyme and protein
4 levels changed as a function of age. Of note is that, in the discussion section of the paper, the
5 authors disclose that injection of TCE 2.0 or 3.0 g/kg i.p. for 5 days resulted in paralytic ileus
6 from TCE exposure as unpublished observations. They note that the rationale for injecting TCE
7 subcutaneously was not only that it did not require an inhalation chamber but also guarded
8 against peritonitis that sometimes occurs following repeated i.p. injection. In terms of
9 comparison with inhalation or oral results, the authors note that the subcutaneous treatment
10 paradigm will result in TCE not immediately being metabolized but retained in the fatty tissue
11 and that after cessation of exposure TCE metabolites continued to be excreted into the urine for
12 more than 2 weeks.
13
14 **2.2.12 NTP 1990**
15
16 *2.2.12.1. 13-week studies*
17
18   The National Toxicology Program (NTP) conducted a 13 weeks study of 7 week old
19 F344/N rats (10 rats per group) that received doses of 125 to 2000 mg/kg (males) [0, 125, 250,
20 500, 1000, or 2000 mg/kg] and 62.5 to 1000 mg/kg (females) [0, 62.5, 125, 250, 500, or 1000
21 mg/kg] TCE via corn oil gavage 5 days per week. For 7-week old B6C3F1mice (n= 10 per
22 group), the dose levels were reported to be 375 to 6000 mg/kg TCE [0, 375, 750, 1500, 3000, or
23 6000 mg/kg]. Animals were exposed via corn oil gavage to TCE that was epichlorhydrin free.
24 All rats were reported to survive the 13 week study, but males receiving 2000 mg/kg exhibited a
25 24% difference in final body weight. However, there was great variation in initial weights
26 between the dose groups with mean initial weights at the beginning of the study reported to 87,
27 88, 92, 95, 101, and 83 grams for the control, 125, 250, 500, 1000, and 2000 mg/kg dose groups
28 in male rats, respectively. This represents a 22 % difference between the highest and lowest
29 initial weights between groups. Thus, changes in final body weight after TCE treatment also
30 reflect differences in starting weights between the groups which in the case of the 500, and 1000
31 mg/kg groups would results in an lower than expected change in weight due to TCE exposure.
32 For female rats the mean initial starting weights were reported to be 81, 72, 74, 75, 73, and 76 g,
33 respectively for the control, 62.5, 125, 250, 500, and 1000 mg/kg dose groups. This represents a
34 ~ 13% difference between initial weights. In the case of female rats the larger mean initial weight
35 in the control group would tend to exaggerate the effects of TCE exposure on final body weight.
36 The authors did not report the variation in initial or final body weights within the dose groups.
37 At the lowest doses for male and female rats body mean weights were reported to be decreased
38 by 6 and 7 % in male and female rats, respectively. Organ weight changes were not reported for
39 rats.
40
41   For male mice, mean initial body weights ranged from 19 to 22 g (~16% difference) and
42 for female mice ranged between 18 and 15 g (20% difference), and thus similar to rats, the final
43 body weights in the groups dose with TCE reflect not only the effects of the compound but also

1 differences in initial weights. For male mice the mean final body weights were reported to be 3
2 to 17% less than controls for the 375 to 3000 mg/kg dose. For female mice the % difference in
3 final body weight was reported to be the same except for the 6000 mg/kg dose group but this
4 lack of difference between controls and treated female mice reflected no change in mice that
5 started at differing weights. Male mice started to exhibit mortality at 1500 mg/kg with 8/10
6 surviving the 1500 mg/kg dose, 3/10 surviving the 3000 mg/kg dose, and none surviving the
7 6000 mg/kg dose of TCE till the end of the study. For females 1 animals out of 10 died in the
8 750, 1500, and 3000 mg/kg dose groups and one surviving the 6000 mg/kg group. In general,
9 the magnitude of increase in TCE exposure concentration was similar to the magnitude of
10 increase in % liver/body weight for the 750 and 1500 mg/kg TCE exposure groups in male
11 B6C3F1 mice and for the 750 to 3000 mg/kg TCE exposure groups in female mice (i.e., a 2-fold
12 increase in TCE exposure resulted in ~ 2-fold increase in % liver/body weight.
13
14 The descriptions of pathology in rats and mice given by this study were not very detailed.
15 For rats only control and high dose rats were examined histologically. For mice only controls
16 and the two highest dose groups were examined histologically. Only mean liver weights were
17 reported with no statistical analyses provided to ascertain quantitative differences between study
18 groups.
19
20 Pathological results were reported to reveal that 6/10 males and 6/10 female rats had
21 pulmonary vasculitis at the highest concentration of TCE. This change was also reported to have
22 occurred in 1/10 control male and female rats. Most of those animals were also reported to have
23 had mild interstitial pneumonitis. The authors report that viral titers were positive during this
24 study for Sendai virus.
25
26 In mice, liver weights (both absolute and as a percent of body weight) were reported to
27 increase with TCE –exposure level. Liver weights were reported to have increased by more than
28 10% relative to controls for males receiving 750 mg/kg or more and for females receiving 1,500
29 mg/kg or more. The most prominent hepatic lesions detected in the mice were reported to be
30 centrilobular necrosis, observed in 6/10 males and 1/10 females administered 6000 mg/kg.
31 "Although centrilobular necrosis was not seen in either males or females administered 3000
32 mg/kg, 2/10 males had multifocal areas of calcifications scattered throughout their livers. These
33 areas of calcification were considered to be evidence of earlier hepatocellular necrosis.
34 Multifocal calcification was also seen in the liver of a single female mouse that survived the
35 6000 mg/kg dosage regime. One female mouse administered 3000 mg/kg also had a
36 hepatocellular adenoma, an extremely rare lesion in female mice of this age (20 weeks)." There
37 appeared to be consistent decrease in liver weight at the lowest dose in both female and male
38 mice after 13 weeks of TCE exposure. Liver weight was increased at exposure concentrations in
39 which there was not increased mortality due to TCE exposure at 13 weeks of TCE exposure.
40
41 Mice data for 13 weeks: mean body and liver weights
42
43 Dose   survival   body weight   liver weight   % liver Wt/body Wt

(mg/kg TCE)		(mean in g)		(mean final in g)	(fold change vs.
control)					
Male		initial	final		
0	10/10	21	36	2.10	5.8
375	10/10	20	35	1.74	5.0 (0.86)
750	10/10	21	32	2.14	6.8 (1.17)
1,500	8/10	19	29	2.27	7.6 (1.31)
3,000	3/10	20	30	2.78	8.5 (1.46)
6,000	0/10	22	-	-	-
Female					
0	10/10	18	26	1.40	5.5
375	10/10	17	26	1.31	5.0 (0.91)
750	9/10	17	26	1.55	5.8 (1.05)
1,500	9/10	17	26	1.80	6.5 (1.18)
3,000	9/10	15	26	2.06	7.8 (1.42)
6,000	1/10	15	27	2.67	9.5 (1.73)

2.2.12.2. 2-year studies

In the 2-year phase of the NTP study, TCE was administered by corn oil gavage to groups of 50 male and 50 female F344/N rats, and B6C3F1 mice. Dosage levels were 500 and 1000 mg/kg for rats and 1000 mg/kg for mice. TCE was administered 5 times a week for 103 weeks and surviving animals were killed between weeks 103 and 107. The same number of animals receiving corn oil gavage served as controls. The animals were 8 weeks old at the beginning of exposure. The focus of this study was to determine if there was a carcinogenic response due to TCE exposure so there was little reporting of non-neoplastic pathology or toxicity.   There was no report of liver weight at termination of the study, only body weight.

The authors reported that there was no increase in necrosis in the liver from TCE exposure in comparison to control mice. In control male mice, the incidence of hepatocellular carcinoma (tumors with markedly abnormal cytology and architecture) was reported to be 8/48 in controls, and 31/50 in TCE-exposed male mice. For females control mice hepatocellular carcinomas were reported in 2/48 of controls and 13/49 of TCE-exposed female mice. Specifically, the authors described liver pathology in mice as follows: "Microscopically the hepatocellular adenomas were circumscribed areas of distinctive hepatic parenchymal cells with a perimeter of normal appearing parenchyma in which there were areas that appeared to be undergoing compression from expansion of the tumor. Mitotic figures were sparse or absent but the tumors lacked typical lobular organization. The hepatocellular carcinomas had markedly abnormal cytology and architecture. Abnormalities in cytology included increased cell size, decreased cell size, cytoplasmic eosinophilia, cytoplasmic basophilia, cytoplasmic vacuolization,

1   cytoplasmic hyaline bodies and variations in nuclear appearance. In many instance, several or all
2   of the abnormalities were present in different areas of the tumor. There were also variations in
3   architecture with some of the hepatocellular carcinomas having areas of trabecular organization.
4   Mitosis was variable in amount and location." The authors report that the non-neoplastic lesion
5   in male mice differing from controls was focal necrosis in 4 vs. 1 animal in the dosed group (8%
6   vs. 2%). There was no fatty metamorphosis in treated male mice vs. 2 animals in control. In
7   female mice there was focal inflammation in 29% vs. 19% of animals (dosed vs. control) and no
8   other changes. Therefore the reported pathological results of this study did not show that the
9   liver was showing signs of toxicity after two years of TCE exposure except for neoplasia.
10
11   For hepatocellular adenomas the incidence was reported to be "7/48 control vs. 14/50
12 dosed in males and 4/48 in control vs. 16/49 dosed female mice."   The administration of TCE to
13 mice was reported to cause increased incidences of hepatocellular carcinomas in males (control,
14 8/48; dosed 31/50: P, 0.001) and in females (control 2/48; dosed 13/49; P<0.005).
15 Hepatocellular carcinomas were reported to metastasize to the lungs in five dosed male mice and
16 one control male mouse, while none were observed in females. The incidences of hepatocellular
17 adenomas were reported to be increased in male mice (control 7/48; dosed 14/50) and in female
18 mice (control 4/48; dosed 16/49; P<0.05). The survival of both low and high dose male rats and
19 dosed male mice was reported to be less than that of vehicle controls with body weight decreases
20 dose dependent. Female mice body weights were comparable to controls. The authors report
21 adjusted rates of 20.6% for control vs. 53.1 % for dosed males for adenoma, 22.1 % control, and
22 92.9 % for carcinoma in males, and liver carcinoma or adenoma adjusted rates of 100%. For
23 female mice the adjusted rates were reported to be 12.5% adenoma for control vs. 55.6% for
24 dosed, and 6.2% control carcinoma vs. 43.9% dosed, with liver carcinoma or adenoma adjusted
25 rates of 18.7% for control vs. 69.7% for dosed.   All of the liver results for male and female
26 mice were reported to be statistically significant. The administration of TCE was reported to
27 cause earlier expression of tumors as the first animals with carcinomas were 57 weeks for TCE-
28 exposed animals and 75 weeks for control male mice.
29
30   In male rats there was no reported treatment related non-neoplastic liver lesions. In
31 female rats a decrease in basophilic cytological change was reported to be of note in TCE treated
32 rats (~ 50% in controls but ~ 5% in TCE treatment groups). However, the authors reported that
33 "the results in male F344/N rats were considered equivocal for detecting a carcinogenic response
34 because both groups receiving TCE showed significantly reduced survival compared to vehicle
35 controls (35/70, 70%; 20/50, 40%; 16/50, 32%) and because 20% of the animals in the high-dose
36 group were killed accidently by gavage error." Specifically 1 male control, 3 low-dose males, 10
37 high-dose males, 2 female controls, 5-low dose females and 5 high-dose female rats were killed
38 by gavage error.
39
40 **2.2.13. NTP 1988**
41
42   The studies described in the NTP 1988 TCE report were conducted "to compare the
43 sensitivities of four strains of rats to diisopropylamine-stabilized TCE." However, the authors

1 conclude "that because of chemically induced toxicity, reduced survival, and incomplete
2 documentation of experimental data, the studies are considered inadequate for either comparing
3 or assessing TCE-induced carcinogenesis in these strains of rats. TCE (more than 99% pure,
4 stabilized with 8ppm diisopropylamine) was administered via corn oil gavage at exposure
5 concentrations of 0, 500 or 1000 mg/kg per day, 5 days per week, for 103 weeks to 50 male and
6 female rats of each strain. The survival of "high-dose male Marshal rats was reduced by a large
7 number of accidental deaths (25 animals were accidentally killed)." However, the report notes
8 survival was decreased at both exposure levels of TCE because of mortality that occurred during
9 the administration of the chemical. The number of animals accidently killed were reported to be:
10 11 male ACI rats at 500 mg/kg, 18 male ACI rats at 1000 mg/kg, 2 vehicle control female ACI
11 rats, 14 female ACI rats at 500 mg/kg , 12 male ACI rats at 1000 mg/kg, 6 vehicle control male
12 August rats, 12 male August rats at 500 mg/kg, 11 male August rats at 1000 mg/kg, 1 vehicle
13 control female August rats, 6 female August rats at 500 mg/kg, 13 male August rats at 1000
14 mg/kg, 2 vehicle control male Marshal rats, 12 male Marshal rats at 500 mg/kg, 25 male Marshal
15 rats at 1000 mg/kg, 3 vehicle control female Marshal rats, 14 female Marshal rats at 500 mg/kg,
16 18 female Marshal rats at 1000 mg/kg, 1 vehicle control male Osborne-Mendel rat, 6 male
17 Osborne-Mendel rats at 500 mg/kg, 7 male Osborne-Mendel rats at 1000 mg/kg, 8 vehicle
18 control female Osborne-Mendel rats, 6 female Osborne-Mendel rats at 500 mg/kg, and 6 female
19 Osborne-Mendel rats at 1000 mg/kg. The age of the rats "when placed on the study" were
20 reported to differ and were for ACI rats (6.5 weeks), August rats (8 weeks), Marshal rats (7
21 weeks), and Osborne-Mendel rats (8 weeks). The ages of sacrifice also varied and were 17-18
22 weeks for the ACI and August rats, and 110-111 weeks for the Marshal rats.
23
24   Results from a 13-week study were briefly mentioned in the report. For the 13-week
25 duration of exposure, groups of 10 male ACI and August rats were administered 0,125, 250, 500,
26 1,000 or 2,000 mg/kg TCE in corn oil gavage. Groups of 10 female ACI and August rats were
27 administered 0, 62.5, 125, 250, 500 or 1,000 mg/kg TCE. Groups of 10 male Marshal rats
28 received 0, 268, 308, 495, 932, or 1,834 mg/kg and groups of female Marshal rats were given 0,
29 134, 153, 248, 466, or 918 mg/kg TCE. With the exception of 3 male August rats receiving 2000
30 mg/kg TCE, all animals survived to the end of the 13-week experimental period. "The
31 administration of the chemical for 13 weeks was not associated with histopathological changes."
32
33   In the two-year study the report noted that there "was no evidence of liver toxicity
34 described as non-neoplastic changes in male ACI rats due to TCE exposure with 4% or less
35 incidence of any lesion in control or treated animals. For female ACI rats, the incidence of fatty
36 metamorphosis was 6% in control vehicle, 9% in low dose TCE, and 13% in high dose TCE
37 groups. There was also a 2%, 11%, and 8% incidence of clear cell change, respectively. A 6%
38 incidence of hepatocytomegaly was reported in vehicle control and 15% incidence in the high
39 dose group." All other desciptors had reported incidences of less than 4%. For August rats there
40 was also little evidence of liver toxicity. In male August rats there was a reported incidence of
41 8%, 4% and 10% focal necrosis in vehicle control, low dose, and high dose, respectively. Fatty
42 metamorphosis was reported to be 8% in control, and 2% and 4% in low and high dose. All
43 other descriptors were reported to be less than 4%. In female August rats, all desciptors of

1   pathology were reported to have a 4% or less incidence except for hepatomegaly which was 10%
2   for vehicle control, 6% for the low dose and 2% for high dose TCE. For male Marshal rats there
3   was a reported 63% incidence of Inflammation, NOS in vehicle control, 12% in low dose and
4   values not recorded at the high dose.   There was a reported 6% and 14% incidence of fatty
5   metamorphosis in control and low dose male rats. Clear cell change was 8% in vehicle with all
6   other values 4% or less. For female Marshal rats all values were 4% or less except for fatty
7   metamorphosis in 6% of vehicle controls. For male Osborne-Mendel rats, there was a reported
8   4%, 10%, and 4% incidence of focal necrosis in vehicle control, low and high dose respectively.
9   For "cytoplasmic change/ NOS," there were reported incidences of 26%, 32% and 27% in
10   vehicle, low dose, and high dose animals, respectively. All other descriptors were reported to be
11   4% or less.   In female Osborne-Mendel rats there was a reported incidence of 10% of focal
12   necrosis at the low dose with all other descriptors reported at 4% or less.

14   Obviously the negative results in this bioassay are confounded by the killing of a large
15   portion of the animals accidently by experimental error. Still, these large exposure
16   concentrations of TCE did not seem to be causing overt liver toxicity in the rat. Organ weights
17   were not reported in this study, which would have been hard to interpret if they had been
18   reported because of the mortality.

20   **2.2.14.   Fukuda et al. 1983**

22   In this 104-week bioassay designed primarily to determine a carcinogenic response,
23   female non-inbred Crj:CD-1 (ICR) mice and female Crj:CD (SD) rats 7 weeks of age were
24   exposed to "reagent grade" TCE at 0, 50, 150, and 450 ppm for 7 hours a day, 5 days a week.
25   During the 2-year duration of the experiment inhalation concentrations were reported to be
26   within 2% of target values. The numbers of animals per group were reported to be 49-50 mice
27   and 49-51 rats at the beginning of the experiment. The impurities in the TCE were reported to be
28   0.128 % carbon tetrachloride benzene, 0.019% epichlorohydrin and 0.019% 1,1,2-
29   trichloroethane. After 107 weeks from commencement of the exposure, surviving animals were
30   reported to be killed and completely necropsied. "Tumors and abnormal organs as well as other
31   major organs were excised and prepared for examination in H&E sections." No other details of
32   the methodologies used for pathological examination of tissues were given including what areas
33   of the liver and number of sections examined by light microscopy.

35   Body weights were not given but the authors reported that "body weight changes of the
36   mice and rats were normal with a normal range of standard deviation." It was also reported that
37   there were no significant differences in average body weight of animals at specified times during
38   the experiments and no significant difference in mortality between the groups of mice. The
39   report includes a figure showing, that for the first 60 weeks of the experiment, there was a
40   difference in cumulative mortality at the 450 ppm dose in ICR mice and the other groups. The
41   authors reported that significantly increased mortalities in the control group of rats compared to
42   the other dosed groups were observed at 85 weeks and after 100 weeks reflecting many deaths
43   during the 81-85 week and 96-100 week periods for control rats. No significant comparable

1 clinical observations were reported to be noted in each group but that major symptoms such as
2 bloody nasal discharge (in rats), local alopecia (in mice and rats), hunching appearance (in mice)
3 and respiratory disorders (in mice and rats) were observed in some animals mostly after 1 year.
4
5   The authors report that "the numbers of different types of tumors were counted and only
6 malignant tumors were counted when both malignant and benign tumors were observed within
7 one organ." They also reported that "all animals were included in the effective numbers except
8 for a few that were killed accidently, severely autolyzed or cannibalized, and died before the first
9 appearance of tumors among the groups." In mice the first tumors were observed at 286 days as
10 thymic lymphoma and most of the malignant tumors appearing later were described as
11 lymphomas or lymphatic leukemias. The incidences of mice with tumors were 37%, 36%, 54%
12 and 52 % in the control, 50 ppm, 150 ppm and 450 ppm groups, respectively, by the end of the
13 experiment. "Tumors of the ovary, uterus, subcutaneous tissue, stomach and liver were observed
14 in the dose groups at low incidences (2-7%) but not in the controls." For the liver, the control,
15 50 ppm, and 150 ppm groups were all reported to have no liver tumors with one animal (2%)
16 having an adenoma at the 450 ppm dose. For rats the first tumor was reported to be observed at
17 410 days and for the incidences of animals with tumors to be 64%, 78%, 66%, and 63% for
18 control, 50 ppm, 150 ppm, and 450 ppm TCE, respectively, by the end of the experiment. Most
19 tumors were distributed in the pituitary gland and mammary gland with other tumors reported at
20 a low incidence of 2-4% with none in the controls. For the liver there were no liver tumors in the
21 control or 150 ppm groups but 1 animal (2%) had a cystic cholangioma in 50 ppm group and one
22 animal (2%) had a hepatocellular carcinoma in the 450 ppm group of rats. No details concerning
23 the pathology of the liver or organ weight changes were given by the authors, including any
24 incidences of hepatomegaly or preneoplastic foci. On note is that in these strains, there were no
25 background liver tumors in either strain, indicative of the relative insensitivity of these strains to
26 hepatocarcinogenicity. However, the carcinogenic potential of TCE was reflected by a number
27 of other tumor sites in this paradigm.
28
29 **2.2.15. Henschler et al. 1980**
30
31   This report focused on the potential carcinogenic response of TCE in mice (NMRI
32 random bred), rats (WIST random bred) and hamsters (Syrian random bred) exposed to 0, 100
33 and 500 ppm TCE for 6 hours/day 5 days/week for 18 months. The TCE used in the experiment
34 was reported to be pure with the exception of trace amounts of chlorinated hydrocarbons,
35 epoxides and triethanolamines (< 0.000025% w/w) and stabilized with 0.0015% triethanolamine.
36 The number of animals in each group was 30 and the ages and initial and final body weights of
37 the animals were not provided in the report. For the period of exposure (8 am – 2 pm), animals
38 were deprived of food and water. The exposure period was for 18 months with mice and
39 hamsters sacrificed after 30 months and rats after 36 months. "Deceased animals" were reported
40 to be autopsied, spleen, liver, kidneys, lungs and heart weighed, and these organs, as well as
41 stomach, CNS, and tumorous tissues, examined in H&E sections.
42

1   Body weight gain was reported to be normal in all species with no noticeable differences
2   between control and exposed groups but data was not shown. However, a "clearly dose-
3   dependent decrease in the survival rate for both male and female mice" was reported to be
4   statistically significant in both sexes and concentrations of TCE with no other significant
5   differences reported in other species. The increase in mortality was more pronounced in male
6   mice, especially after 50 weeks of exposure. Hence the opportunity for tumor development was
7   diminished due to decreased survival in TCE treated groups. No organ weights were provided
8   for the study due to the design, in which at considerable period of time occurred between the
9   cessation of exposure and the sacrifice of the animals and liver weights changes due to TCE may
10   have been diminished with time. For the 30 autopsied male mice in the control group, 1
11   hepatocellular adenoma and 1 hepatocellular carcinoma was reported. Whether they occurred in
12   the same animal cannot be determined from the data presentation. In the 29 animals in the100
13   ppm TCE exposure group 2 hepatocellular adenomas and 1 mesenchymal liver tumor were
14   reported but no hepatocellular carcinomas also without a determination as whether they occurred
15   in the same animal or not. In the 30 animals autopsied in the 500 ppm exposure group no liver
16   tumors were reported. In female mice, of the 29 animals autopsied in the control group, 30
17   animals autopsied in the 100 ppm group, and the 28 animals autopsied in the 500 ppm group,
18   there were also no liver tumors reported.
19
20   In both the 100 ppm and 500 ppm exposure groups, of male mice especially, low
21   numbers of animals studied, abbreviated TCE exposure duration, and lower numbers of animals
22   surviving to the end of the experiment, limit the power of this study to determine a treatment-
23   related difference in liver carcinogenicity.   As discussed in section 2.3.2. below, the use of an
24   abbreviated exposure regime or study duration and low numbers of animals examined limits the
25   power of a study to detect a treatment-related response. The lack of any observed background
26   liver tumors in the female mice and a very low background level of 2 tumors in the male mice
27   are indicative of a low sensitivity to detect liver tumors in this paradigm which may have
28   occurred either through its design or a low sensitivity of mouse strain used for this endpoint.
29   However, the carcinogenic potential of TCE in mice was reflected by a number of other tumor
30   sites in this paradigm.
31
32   For rats and hamsters the authors reported "no dose-related accumulation of any kind of
33   tumor in either sex of these species." For male rats there was only 1 hepatocellular
34   adenoma reported at 100 ppm in the 30 animals autopsied and no carcinomas. For female rats
35   there were no liver tumors reported in control animals but, more significantly, at 100 ppm there
36   was 1 adenoma and 1 cholangiocarcinoma reported at 100 ppm and at 500 ppm 2
37   cholangioadenomas. Although not statistically significant, the occurrence of this relatively rare
38   biliary tumor was observed in both TCE dose groups in female rats. The difference in survival,
39   as reported in mice, did not affect the power to detect a response in rats, but the low numbers of
40   animals studied, abbreviated exposure duration and apparent low sensitivity to detect a
41   hepatocarcinogenic response suggest a study of low power. Nevertheless, the occurrence of
42   cholangioadenomas and 1 cholangiocarcinoma in female rats after TCE treatments is of concern,
43   especially given the relationship in origin and proximity of the bile and liver cells and the low

1   incidence of this tumor. For hamsters the low background rate of tumors of any kind suggests
2   that in this paradigm, the sensitivity for detection of this tumor is relatively low.
3
4   **2.2.16. Maltoni et al. 1986**
5
6   The report by Maltoni et al. (1986) included a series of "systematic and integrated
7   experiments (BT 301, 302, 303, 304, 304bis, 305, 306 bis) started in sequence, testing TCE by
8   inhalation and by ingestion."   The first experiment (BT 301) was begun in 1976 and the last in
9   1983 with this report representing the complete report of the findings and results of project. The
10   focus of the study was detection of a neoplastic response with only a generalized description of
11   tumor pathology phenotype given and no reporting of liver weight changes induced by TCE
12   exposure.
13
14   In experiment BT 301, TCE was administered in male and female Sprague-Dawley rats
15   (13 weeks at start of experiment) via olive oil gavage at control, 50 mg/kg or 250 mg/kg
16   exposure levels for 52 weeks (4-5 days weekly).   The animals (30 male, 30 female for each dose
17   group) were examined during their lifetime. In experiment BT 302, male and female Sprague-
18   Dawley rats (13 weeks old at start of the experiment) were exposed to TCE via inhalation at 0,
19   100, and 600 ppm, 7 hours a day, 5 days a week, for 8 weeks. The animals (90 animals in each
20   control group, 60 animals in each 100 ppm group, and 72 animals in each 600 ppm group) were
21   examined during their lifetime. In experiment BT 304, male and female Sprague Dawley rats
22   (12 weeks old at start of the experiment) were exposed TCE via inhalation at 0, 100, 300, and
23   600 ppm 7 hours a day, 5 days a week, for 104 weeks. The animals (95 male, 100 female rats
24   control groups, 90 animals in each 100 ppm group, 90 animals in each 300 ppm group, and 90
25   animals in each 600 ppm group) were examined during their lifetime. In experiment BT304bis,
26   male and female Sprague Dawley rats (12 weeks old at start of the experiment) were exposed to
27   TCE via inhalation at 0, 100, 300, and 600 ppm for 7 hours a day, 5 days a week, for 104 weeks.
28   The animals (40 male, 40 female rats control groups, 40 animals in each 100 ppm group, 40
29   animals in each 300 ppm group, and 40 animals in each 600 ppm group) were examined during
30   their lifetime.
31
32   In experiment BT 303, Swiss mice (11 weeks old at the start of the experiment) were
33   exposed to TCE via inhalation in for 8 weeks using the same exposure concentrations as for
34   experiment BT 302. The animals (100 animals in each control group, 60 animals in the 100 ppm
35   exposed group, and 72 animals in each 600 ppm group) were examined during their lifetime. In
36   experiment BT 305, Swiss mice (11 weeks old at the start of the experiment) were exposed to
37   TCE via inhalation in for 78 weeks, 7 hours a day, 5 days a week. The animals (90 animals in
38   each control group, 90 animals in the 100 ppm exposed group, 90 animals in the 300 ppm group,
39   and 90 animals in each 600 ppm group) were examined during their lifetime. In experiment BT
40   306, B6C3F1 mice (from NCI source) (12 weeks old at the start of the experiment) were exposed
41   to TCE via inhalation in for 78 weeks, 7 hours a day, 5 days a week. The animals (90 animals in
42   each control group, 90 animals in the 100 ppm exposed group, 90 animals in the 300 ppm group,
43   and 90 animals in each 600 ppm group) were examined during their lifetime. In experiment BT

1   306bis B6C3F1 mice (from Charles River Laboratory as source) (12 weeks old at the start of the
2   experiment) were exposed to TCE via inhalation for 78 weeks, 7 hours a day, 5 days a week. The
3   animals (90 animals in each control group, 90 animals in the 100 ppm exposed group, 90 animals
4   in the 300 ppm group, and 90 animals in each 600 ppm group) were examined during their
5   lifetime.
6
7   In all experiments, TCE was supplied tested and reported by the authors of the study to
8   be was highly purified and epoxide free with butyl-hydroxy-toluene at 20 ppm used as a
9   stabilizer.   Extra-virgin olive oil was used as the carrier for ingestion experiments and was
10   reported to be free of pesticides. The authors describe the treatment of the animals and running
11   of the facility in detail and report that: "Animal rooms were cleaned every day and room
12   temperature varied from 19 degrees to 22 degrees and was checked 3 times daily. Bedding was
13   changed every two days and cages changes and washed once weekly. The animals were handled
14   very gently and, therefore, were neither aggressive nor nervous. Concentrations of TCE were
15   checked by continuous gas-chromatographic monitoring. Treatment was performed by the same
16   team. In particular, the same person carried out the gavage of the same animals. This is
17   important, since animals become accustomed to the same operators. The inhalation chambers
18   were maintained at 23 ± 2 degrees C and $50 \pm 10\%$ relative humidity. Ingestion from Monday to
19   Friday was usually performed early in the morning. The status and behavior of the animals were
20   examined at least three times daily and recorded. Every two weeks the animals were submitted
21   to an examination for the detection of the gross changes, which were registered in the
22   experimental records. The animals which were found moribund at the periodical daily inspection
23   were isolated in order to avoid cannibalism. The animals were weight every two weeks during
24   treatment and then every eight weeks. Animals were kept under observation until spontaneous
25   death. A complete necropsy was performed. Histological specimens were fixed in 70% ethyl
26   alcohol. A higher number of samples was taken when particular pathological lesions were seen.
27   All slides were screened by a junior pathologist and then reviewed by a senior pathologist. The
28   senior pathologist was the same throughout the entire project. Analysis of variance was used for
29   statistical evaluation of body weights. Results are expressed as means and standard deviations.
30   Survival time is evaluated using the Kruskal-Wallis test. For different survival rates between
31   groups, the incidence of lesions is evaluated by using the Log rank test. Non-neoplastic,
32   preneoplastic, and neoplastic lesions were evaluated using the Chi-square of Fisher' exact test.
33   The effect of different doses was evaluated using the Cochran-Armitage test for linear trends in
34   proportions and frequencies." The authors state that: "Although the BT project on TCE was
35   started in 1976 and most of the experiments were performed from the beginning of 1979, the
36   methodological protocol adopted substantially met the requirements of the Good Laboratory
37   Practices Act." Finally, it was reported that "the experiments ran smoothly with no accidents in
38   relation to the conduct of the experiment and the health of the animals, apart from an excess in
39   mortality in the male B6C3F1 mice of the experiment BT 306, due to aggressiveness and
40   fighting among the animals." This is in contrast to the description of the gavage studies
41   conducted by NTP (NTP 1990, 1988) in which gavage error resulted in significant loss of
42   experimental animals. Questions have been raised about the findings, experimental conditions,
43   and experimental paradigm of the European Ramazzini Foundation (ERF) from which the

1   Maltoni et al (1986) experiments were conducted (EFSA, 2006). However, these concerns were
2   addressed by Caldwell et al. (2008a), who concluded that the ERF bioassay program produced
3   credible results that were generally consistent with those of NTP
4   In regards to effects of TCE exposure on survival, "a nonsignificant excess in mortality
5   correlated to TCE treatment was observed only in female rats (treated by ingestion with the
6   compound) and in male B6C3F1 mice. In B6C3F1 mice of the experiment BT 306 bis, the
7   excess in mortality in treated animals was higher (p < 0.05 after 40 weeks) but was not dose
8   correlated. No excess in mortality was observed in the other experiments." The authors reported
9   that "no definite effect of TCE on body weight was observed in any of the experiments, apart
10   from experiment BT 306 bis, in which a slight non-dose correlated decrease was found in
11   exposed animals."
12
13   In mice, "hepatoma" was the term used by the authors of these studies to describe all
14   malignant tumors of hepatic cells, of different sub-histotypes, and of various degrees of
15   malignancy. The authors reported that the hepatomas induced by exposure to TCE "may be
16   unique or multiple, and have different sizes (usually detected grossly at necropsy). Under
17   microscopic examination these tumors proved to be of the usual type observed in Swiss and
18   B6C3F1 mice, as well as in other mouse strains, either untreated or treated with
19   hepatocarcinogens. They frequently have medullary (solid), trabecular, and pleomorphic
20   (usually anaplastic) patterns. The hepatomas may produce distant metastases, more frequently in
21   the lungs."
22
23   In regard to the induction of "hepatomas" by TCE exposure, the authors report that in
24   Swiss mice exposed to TCE by inhalation for 8 weeks (BT303), the percentage of animals with
25   hepatomas was 1.0% in male mice and 1.0% in female mice in the control group (n= 100 for
26   each gender). For animals exposed to 100 ppm TCE, the percentage in female mice was 1.7%
27   and male mice 5.0% (n = 60 for each gender). For animals exposed to 600 ppm TCE, the
28   percentage in female mice was 0% and in male mice 5.5% (n= 72 for each gender).   The
29   relatively larger number of animals used in this bioassay, in comparison to NTP standard assays,
30   allows for a greater power to detect a response. It is also apparent from these results that Swiss
31   mice in this experimental paradigm are a "less sensitive" strain in regard to spontaneous liver
32   cancer induction over the lifetime of the animals. These results suggest that 8 weeks of TCE
33   exposure via inhalation at 100 ppm or 600 ppm may have been associated with a small increase
34   in liver tumors in male mice in comparison to concurrent controls.
35
36   In Swiss mice exposed to TCE via inhalation for 78 weeks (BT 305), the percentage of
37   animals with hepatomas was reported to be 4.4% in male mice and 0% in female mice in the
38   control group (n= 90 for each gender). For animals exposed to 100 ppm TCE, the percentage in
39   female mice was reported to be 0% and male mice 2.2% (n = 90 for each gender). For animals
40   exposed to 300 ppm TCE, the percentage in female mice was reported to be 0% and in male
41   mice 8.9% (n= 90 for each gender). For animals exposed to 600 ppm TCE, the percentage in
42   female mice was reported to be 1.1% and in male mice 14.4%. As with experiment BT303, there
43   is a consistency in the relatively low background level of hepatomas reported for Swiss mice in

1   this paradigm. After 78 weeks of exposure there appears to be a dose-related increase in
2   hepatomas in male but not female Swiss mice via inhalation exposure.
3
4   In B6C3F1 mice exposed to TCE by inhalation for 78 weeks (BT306) the percentage of
5   animals with hepatomas was reported to be 1.1% in male mice and 3.3 % in female mice in the
6   control group (n= 90 for each gender). For animals exposed to 100 ppm TCE, the percentage in
7   female mice was reported to be 4.4% and in male mice 1.1% (n = 90 for each gender). For
8   animals exposed to 300 ppm TCE, the percentage in female mice was reported to be 3.3% and in
9   male mice 4.4% (n= 90 for each gender). For animals exposed to 600 ppm TCE, the percentage
10   in female mice was reported to be 10.0% and in male mice 6.7%. This was the experimental
11   group with excess mortality in the male group due to fighting. The excess mortality could have
12   affected the results. The authors do report that there was a difference in the percentage of males
13   bearing benign and malignant tumors that was due to early mortality among males in experiment
14   BT306. It is unexpected for the liver cancer incidence to be less in male mice than female mice
15   and not consistent with the results reported for the Swiss mice.
16
17   In B6C3F1 male mice exposed to TCE via inhalation (BT 306 bis) the percentage of
18   animals with hepatomas was reported to be 18.9% in male mice in the control group (n= 90).
19   For animals exposed to 100 ppm TCE, the percentage in male mice was reported to be 21.1% (n
20   = 90). For animals exposed to 300 ppm TCE, the percentage in male mice was reported to be
21   30.0 % (n= 90). For animals exposed to 600 ppm TCE, the percentage in male mice was
22   reported to be 23.3%. This experiment did not examine female mice. The authors do report a
23   decrease in survival in mice from this experiment that could have affected results. It is apparent
24   from the BT 306 and BT 306 bis experiments that the background level of liver cancer was
25   significantly different in male mice, although they were supposed to be of the same strain. The
26   finding of differences in response in animals of the same strain but from differing sources has
27   also been reported in other studies for other endpoints (see Section 3.1.2., below).
28
29   The authors reported 4 liver angiosarcomas: 1 in an untreated male rat (BT 304); 1 in a
30   male and 1 in a female rat exposed to 600 ppm TCE for 8 weeks (experiment BT302); and 1 in a
31   female rat exposed to 600 ppm TCE for 104 weeks (BT 304). The authors conclude that "the
32   tumors observed in the treated animals cannot be considered to be correlated to TCE treatment,
33   but are spontaneously arising. These findings are underlined because of the extreme rarity of this
34   tumor in control Sprague Dawley rats, untreated or treated with vehicle materials. The
35   morphology of these tumors is of the liver angiosarcoma type produced by vinyl chloride in this
36   strain of rats."
37
38   In rats treated for 104 weeks, TCE was reported to not affect the percentages of animals
39   bearing benign and malignant tumor and of animals bearing malignant tumors. Moreover, it did
40   not affect the number of total malignant tumors per 100 animals. This study did not report a
41   treatment related increase in liver cancer in rats. The report only explicitly described positive
42   findings so it is assumed that there were no increases in "hepatomas" in rat liver associated with
43   TCE treatment. The authors concluded that "under the tested experimental conditions, the

evidence of TCE (without epoxide stabilizer) carcinogenicity, gives the result of TCE treatment-related hepatomas in male Swiss and B6C3F1 mice. A borderline increased frequency of hepatomas was also seen after 8 weeks of exposure in male Swiss mice." Thus, the increase in liver tumors in both strains of mice exposed to TCE via inhalation reported in this study is consistent with the gavage results from the NTP 1990 study in B6C3F1 mice, where male mice had a higher background level and greater response from TCE exposure than females.

2.2.17. Maltoni et al. 1988

This report was an abbreviated description of an earlier study (Maltoni et al 1986) focusing on the identification of a carcinogenic response in rats and mice by chronic TCE exposure.

2.2.18. Van Duuren et al. 1979

This study exposed male and female non-inbred HA:ICR Swiss mice at 6-8 weeks of age to distilled TCE with no further descriptions of purity. Gavage feeding of TCE was once weekly in 0.1 ml trioctanoin. Neither initial or final body weights were reported by the authors. The authors reported that, at the termination of the experiments or at death, animals were completely autopsied with specimens of all abnormal-appearing tissues and organs excised for histopathologic diagnosis. Tissues from the stomachs, livers and kidneys were reported to be taken routinely for the intragastric feeding experiments. Tissues were reported to be stained for H&E for pathologic examination, but no further description of the lobe(s) of the liver examined or the sections examined was provided by the authors. Results were as only reported the no of mice with forestomach tumors 0.5 mg/ mouse of TCE treatment given once a week in 0.1 ml trioctanoin. Mouse body weights were not given so the dose in mg/kg for the mice cannot be ascertained. The protocol used in this experiment kept the mg/mouse constant with a 1 week dosing schedule so that as the mice increased weight with age, the dose as a function of body weight was decreased. The days on test were reported to be 622 for 30 male and female mice. 2 male and 1 female mice were reported as having forestomach tumors. For 30 mice treated with trioctanoin alone the number of forestomach tumors was reported to be zero. For mice with no TCE treatment, 5 of 100 male mice were reported to have forestomach tumors and of 8 of 60 female mice were reported to have forestomach tumors for 636 and 649 days on test.   No results for liver were presented by the authors by the intragastric route of administration including background rates of the incidences of liver tumors or treatment results. The authors note that except for repeated skin applications of certain chemicals, no significant difference between the incidence of distant tumors in treated animals compared with no-treatment and vehicle control groups was noted. Given the uncertainties in regard to dose, the once-a week dosing regime, the low number of animals tested with resulting low power, and the lack of reporting of experimental results, the ability to use the results from this experiment in regard to TCE carcinogenicity is very limited.

1 **2.2.19. NCI 1976**
2
3   This bioassay was "initiated in 1972 according to the methods used and widely accepted
4 at that time" with the design of carcinogenesis bioassays having "evolved since then in some
5 respects and several improvements" having been developed.   The most notable changes reported
6 in the foreward of the report are changes "pertaining to preliminary toxicity studies, numbers of
7 controls used, and extent of pathological examination."   Industrial grade TCE was tested (99%
8 TCE, 0.19% 1,2,-epoxybutane, 0.04%v ethyl acetate, 0.09% epichlorhydrin, 0.02% N-methyl
9 pyrrole, and 0.03% diisobutylene) with rats and mice exposed via gavage in corn oil 5
10 times/week for 78 weeks using 50 animals per group at 2 doses with both sexes of Osborne-
11 Mendel rats and B6C3F1 mice. However for control groups only 20 of each sex and species
12 were used. Rats were killed after 110 weeks and mice after 90 weeks. Rats and mice were
13 initially 48 and 35 days of age, respectively, at the start of the experiment with control and
14 treated animals born within 6 days of each other. Initial weight ranges were reported as ranges
15 for treated and control animals of 168-229 g for male rats, 130-170 g for female rats, 11-22 g for
16 male mice, and 11-18 g for female mice. Animals were reported to be "randomly assigned to
17 treatment groups so that initially the average weight in each group was approximately the same."
18 Mice treated with TCE were reported to be "maintained in a room housing other mice being
19 treated with one of the following 17 compounds: 1,1,2-2-tetrachloroethane, chloroform, 3-
20 chloropropene, chloropicrin, 1,2-dibromochloropropane, 1,2, dibromoethane, ethylene
21 dichloride, 1,1-diochloroethane, 3-sulfolene, idoform, methyl chloroform, 1,1,2-trichloroethane,
22 tetrachloroethylene, hexachloroethane, carbon disulfide, trichlorofluoromethane, and carbon
23 tetrachloride. Nine groups of vehicle controls and 9 groups of untreated controls were also
24 housed in this same room." The authors note that "TCE-treated rats and their controls were
25 maintained in a room housing other rats being treated with one of the following compounds:
26 dibromochloropropane, ethylene dichloride, 1,1-dichloroethane, and carbon disulfide. Four
27 groups of vehicle-treated controls were in the same room." Thus, there was the potential of co-
28 exposure to a number of other chemicals, especially for the mice, resulting from exhalation in
29 treated animals housed in the same room, including the control groups, as noted by the authors.
30 The authors also noted that "samples of ambient air were not tested for presence of volatile
31 materials" but state that "although the room arrangement is not desirable as is stated in the
32 Guidelines for Carcinogen Bioassay in Small Rodents, there is not evidence the results would
33 have been different with a single compound in a room."
34
35   The initial doses of TCE for rats were reported to be 1300 and 650 mg/kg. However,
36 these levels were changed based on survival and body weight data "so that the time-weighted
37 average doses were 549 and 1097 mg/kg for both male and female rats."   For mice, the initial
38 doses were reported to be 1000 and 2000 mg/kg for males and 700 and 1400 mg/kg for females.
39 The "doses were increased so that the time weighted average doses were 1169 mg/kg and 2339
40 mg/kg for male mice and 869 and 1739 mg/kg for female mice." The authors reported that signs
41 of toxicity, including reduction in weight, were evident in treated rats which, along with
42 increased mortality, "necessitated a reduction in doses during the test." In contrast "very little
43 evidence of toxicity was seen in mice, so doses were increased slightly during the study." Doses

1 were "changed for the rats after 7 and 16 weeks of treatment, and for the mice after 12 weeks."
2 At 7 weeks of age, male and female rats were dosed with 650mg/kg TCE, at 14 weeks they were
3 dosed with 750 mg/kg TCE, and at 23 weeks of age 500 mg/kg TCE. For the high exposure
4 level, the exposure concentrations were 1300 mg/kg, 1500 mg/kg, and 1000 mg/kg TCE,
5 respectively, for the same changes in dosing concentration. For rats the percentage of TCE in
6 corn oil remained constant at 60%. For female mice, the TCE exposure at the beginning of
7 dosing was 700 mg/kg TCE (10% in corn oil) at 5 weeks of age for the "lower dose" level. The
8 dose was increased to 900 mg/kg day (18% in corn oil) at 17 weeks of age and maintained until
9 83 weeks of age.   For male mice, the TCE exposure at the beginning of dosing was 1000 mg/kg
10 TCE (15% in corn oil) at 5 weeks of age for the "lower dose" level. At 11 weeks, the level of
11 TCE remained the same but the percentage of TCE in corn oil was reduced to 10%. The dose
12 was increased to 1200 mg/kg day at 17 weeks of age (24% in corn oil) and maintained until 83
13 weeks of age. For the "higher dose," the TCE exposure at the beginning of dosing was 1400
14 mg/kg TCE (10% in corn oil) at 5 weeks of age in female mice. At 11 weeks of age the exposure
15 level of TCE was kept the same but the percentage of TCE in corn oil increased to 20%. By 17
16 weeks of age the exposure concentration of TCE in corn oil was increased to 1800 mg/kg (18%
17 in corn oil) in female mice.   For the "higher dose" in male mice, the TCE exposure at the
18 beginning of dosing was 2000 mg/kg (15% in corn oil) which was maintained at 11 weeks in
19 regard to TCE administered but the % of TCE corn oil was increased to 20%. For male mice the
20 exposure concentration was increased to 2400 mg/kg (24% in corn oil).   For all of the mice
21 treatment continued on a 5 days/week schedule of oral gavage dosing throughout the timecourse
22 of treatment (78 weeks of treatment). Thus, not only did the total dose administered to the
23 animals change, but the volumes of vehicle in which TCE was administered changed throughout
24 the experiment.
25
26   The authors stated that at 37 weeks of age, "To help assure survival until planned
27 termination the dosing schedule was changed for rats to a cycle of 1 week of no treatment
28 followed by 4 weeks of treatment." for male and female rats. Thus, the duration of exposure in
29 rats was also changed. All lobes of the liver were reported to be taken including the free margin
30 of each lobe with any nodule or mass represented in a block 10 x 5 x 3 mm cut from the liver and
31 fixed in a marked capsule.
32
33   Body weights (mean ± Std) were reported to be 193 ± 15.0 g (n=20), 193 ± 15.8 g
34 (n=50), and 195 ± 16.7 g (n=50) for control, low, and high dose male rats at initiation of the
35 experiment. By 1 year of exposure (50 weeks), 20/20 control male rats were still alive to be
36 weighed, 42/50 of the low dose rats were alive and 34/50 of high dose rats were still alive. The
37 body weights of those remaining were decreased by 6.2% and 17% in the low and high dose
38 animals in comparison with the controls. For female rats, the mean body weights were reported
39 to be 146 ± 11.4 g (n= 20), 144 ± 11.0 g (n= 50), and 144 ± 9.5 g (n=50) for control, low, and
40 high dose female rats at initiation of the experiment.   By 1 year of exposure (50 weeks), 17/20
41 control female rats were still alive, 28/50 low dose and 39/50 of the high dose rats were alive.
42 The body weights of those remaining were decreased by 25% and 30% in the low and high dose
43 animals in comparison with the controls. For male mice the initial body weights were 17 ± 0.5 g

1   (n=20), 17 ± 2.0 g (n=50), and 17 ± 1.1 g (n=50) for control, low and high doses. By 1 year of
2   exposure (50 weeks), 18/20 control male mice were still alive, 47/50 or the low dose, and 34/50
3   of the high dose groups were still alive. The body weights of those remaining were unchanged in
4   comparison to controls. For female mice the initial body weights were 14 ± 0.0 g (n=20), 14 ±
5   0.6 g (n=50), and 14 ± 0.7 g (n=50) for control, low and high doses. By 1 year of exposure (50
6   weeks), 18/20 control male mice were still alive, 45/50 or the low dose, and 41/50 of the high
7   dose groups were still alive. The body weights of those remaining were unchanged in
8   comparison to controls.
9
10   A high proportion of rats were reported to die during the experiment with 17/20 control,
11 42/50 low dose, and 47/50 high dose animals dying prior to scheduled termination. For female
12 rats, 12/20 control, 35/48 low dose, and 37/50 high dose animals were reported to die before
13 scheduled termination with two low dose females reported to be missing and not counted in the
14 denominator for that group. The authors reported that earlier death was associated with higher
15 TCE dose. A decrease in the percentage of tumor-bearing animals was reported to be lower in
16 treated animals and attributed by the authors to be likely related to the decrease in their survival.
17 A high percentage of respiratory disease was reported to be observed among the rats without any
18 apparent difference in the type, severity, or morbidity as to sex or group. The authors reported
19 that "no significant toxic hepatic changes were observed" but no other details regarding results in
20 the liver of rats. Carbon tetrachloride was administered to rats as a positive control. A low
21 incidence of both hepatocellular carcinoma and neoplastic nodule was reported to be found in
22 both colony controls (1/99 hepatocellular carcinoma and 0/99 neoplastic nodule in male rats and
23 0/98 hepatocellular carcinoma and 2/98 neoplastic nodules in female rats) and carbon-
24 tetrachloride-treated rats. Hepatic adenomas were included in the description of neoplastic
25 nodules in this study with the diagnosis of hepatocellular carcinoma to be "based on the presence
26 of less organized architecture and more variability in the cells comprising the neoplasms."
27
28   The authors reported that "increased mortality in treated male mice appears to be related
29 to the presence of liver tumors." For mice both male and female mice the incidences of
30 hepatocellular carcinoma were reported to be high from TCE treatment with 1/20 in age matched
31 controls, 26/50 in low dose and 31/48 in high dose males. Colony controls for male mice were
32 reported to be 5/77 for vehicle and 5/70 for untreated mice.   For females mice hepatocellular
33 carcinomas were reported to be observed in 0/20 age matched controls, 4/50 low dose, and 11/47
34 high-dose female mice. Colony controls for female mice were reported to be 1/80 for vehicle
35 and 2/75 for untreated mice. In male mice, hepatocellular carcinomas were reported to be
36 observed early in the study with the first seen at 27 weeks. Hepatocellular carcinomas were not
37 observed so early in low dose male or female mice.
38
39   The diagnosis of hepatocellular carcinoma was reported to be based on histologic
40 appearance and the presence of metastasis especially to the lung with not other lesions
41 significantly elevated in treated mice.   The tumors were reported to be "varied from those
42 composed of well differentiated hepatocytes in a relatively uniform trabecular arrangement to
43 rather anaplastic lesions in which mitotic figures occurred in cells which varied greatly in size

1 and tinctorial characteristics. Many of the tumors were characterized by the formation of
2 relatively discrete areas of highly anaplastic cells within the tumor proper which were, in turn,
3 surrounded by relatively well differentiated neoplastic cells. In general, various arrangements of
4 the hepatocellular carcinoma occurred, as described in the literature, including those with an
5 orderly cord-like arrangement of neoplastic cells, those with a pseudoglandular pattern
6 resembling adenocarcinoma, and those composed of sheets of highly anaplastic cells with
7 minimal cord or gland-like arrangement.   Multiple metaplastic lesions were observed in the
8 lung, including several neoplasms which were differentiated and relative benign in appearance."
9 The authors noted that almost all mice treated with carbon tetrachloride exhibited liver tumors
10 and that the "neoplasms occurring in treated [sic carbon tetrachloride treated] mice were similar
11 in appearance to those noted in the trichloroethylene-treated mice." Thus, phenotypically this
12 study reported that the liver tumors induced in mice by TCE were heterogeneous and typical of
13 those arising after carbon tetrachloride administration. The descriptions of liver tumors in this
14 study and the tendency of metastasis to the lung are similar to the descriptions provided by
15 Maltoni et al., (1986) for TCE-induced liver tumors in mice via inhalation.
16
17   In terms of non-cancer pathology of the liver, 1 control male rat was reported to display
18 fatty metamorphosis of the liver at 102 weeks. However for the low dose, 3 male rats were
19 reported to display fatty metamorphosis (90, 110, and 110 weeks), 2 rats to display cystic
20 inflammation (76, 110 weeks), and one rat to display general inflammation (110 weeks). At the
21 high dose, 6 rats were reported to display fatty metamorphosis (12, 35, 49, 52, 52, and 58
22 weeks), 1 rat was reported to display cytomegaly (42 weeks), 2 rats were reported to display
23 centrilobular degeneration (53 and 58 weeks), 1 rats to display diffuse inflammation (62 weeks),
24 1 rat to display congestion (week 12), and 5 rats to display angiectasis or abnormally enlarged
25 blood vessels which can be manifested by hyperproliferation of endothelial cells and dilatation of
26 sinusoidal spaces (35, 42, 52, 54, and 65 weeks). One control female rat was reported o display
27 fatty metaphorphosis of the liver at 110 weeks, and one control female rats to display
28 "inflammation" of the liver at 110 weeks. Of the TCE dosed female rats, only 1 high dose
29 female rat displayed fatty metaphorphosis at week 96. Thus for male rats, there was liver
30 pathology present in some rats due to TCE exposure examined from 12 weeks to a year at their
31 time of their premature death. For mice the liver pathology was dominated by the presence of
32 hepatocellular carcinoma with additional hyperplasia noted in 2 mice of the high dose male and
33 female groups and 1 or less mouse exhibiting hyperplasia in the control or low dose groups.
34
35   The authors note that "while the absence of a similar effect in rats appears most likely
36 attributable to a difference in sensitivity between the Osborne-Mendel rat and B6C3F1 mouse,
37 the early mortality of rats due to toxicity must also be considered." The conclude that "the test in
38 rats is inconclusive: large numbers of rats died prior to planned termination; in addition, the
39 response of this rat strain to the hepatocarcinogenicity of the positive control compound, carbon
40 tetrachloride, appeared relatively low."   Finally, the authors note that "while the results obtained
41 in the present bioassay could possibly have been influenced by an impurity in the TCE used, the
42 extremely low amounts of impurities found make this improbable."
43

1   **2.2.20. Herren-Freund et al. 1987**
2
3   This study was gave results primarily in initiated male B6C3 F1 mice that were also
4   exposed to TCE metabolites in drinking water for 61 weeks. However in Table 1 of the report,
5   results were given for mice that received no initiator but were given 40 mg/l TCE or 2 g/l NaCL
6   as control. The mice were reported to be 28 days of age when placed on drinking water
7   containing TCE. The authors reported that concentrations of TCE fell by about ½ at the 40 mg/l
8   dose of TCE during the twice a week change in drinking water solution. For control animals
9   (n=22) body weight at termination was reported to be 32.93 ± 0.54 g, and liver weight was 1.80
10   ± 0.05 g, % liver/body weight was 5.47 ± 0.16%. For TCE treated animals (n=32), body weight
11   at termination was reported to be 35.23 ± 0.66 g, and liver weight was 1.97 ± 0.10 g, %
12   liver/body weight was 5.57 ± 0.24%. Thus, hepatomegaly was not reported for this paradigm at
13   this time of exposure. The study reported that for 22 control animals, the prevalence of
14   adenomas was 2/22 animals (or 9%) with the mean number of adenomas per animal to be 0.09 ±
15   0.06 (SEM). The prevalence of carcinomas in the control group was reported to be 0/22. For 32 animals
16   exposed to 40 mg/l TCE, the prevalence of adenomas was 3/32 animals (or 9%) with the mean number of
17   adenomas per animal to be 0.19 ± 0.12 (SEM). The prevalence of animals with hepatocellular
18   carcinomas was 3/32 animals (or 9%) with the mean number of hepatocellular carcinomas to be 0.10 ±
19   0.05 (SEM). Thus, similar to the acute study of Tucker et al. (1982), significant loss of TCE is a
20   limitation for trying to evaluate TCE hazard in drinking water. However, despite difficulties in
21   establishing accurately the dose received, an increase in adenomas per animal and an increase in
22   the number of animals with hepatocellular carcinomas were reported to be associated with TCE
23   exposure after 61 weeks of exposure. Also of note is that the increase in tumors was reported
24   without significant increases in hepatomegaly at the end of exposure. The authors did not report
25   these increases in tumors as being significant but did not do a statistical test between TCE
26   exposed animals without initiation and control animals without initiation. The low numbers of
27   animal tested limits the statistical power to make such a determination. However for carcinomas,
28   there was none reported in controls but 9% of TCE-treated mice had hepatocellular carcinomas.
29
30   **2.2.21. Anna et al. 1994:**
31
32   The report focused on presenting incidence of cancer induction after exposure to TCE or
33   its metabolites and included a description of results for male B6C3F1 mice (8 weeks old at the
34   beginning of treatment) receiving 800 mg/kg/day TCE via gavage in corn oil, 5 days/week for 76
35   weeks. There was very limited reporting of results other than tumor incidence. There was no
36   reporting of liver weights at termination of the experiment. Although the methods section of the
37   report gives 800 mg/kg/day as the exposure level, Table 1 in the results section reports that TCE
38   was administered at 1700 mg/kg/day. This could be a typographical error in the table as a
39   transposition with the dose of "perc" administered to other animals in the same study. The
40   methods section of the report states that the authors based their dose in mice that used in the
41   1990 NTP study. The NTP study only used a1000 mg/kg/day in mice suggesting that the table is
42   mislabeled and suggests that the actual dose is 800 mg/kg/day in the Anna et al., 1994 study. All
43   treated mice were reported to be alive after 76 weeks of treatment. For control animals, 10

1   animals exposed to corn oil, and 10 untreated controls were killed in a 9-day period. The
2   remaining controls were killed at 96, 103, 134 weeks of treatment. Therefore, the control group
3   (all) contains a mixed group of animals that were sacrificed from 76 -134 weeks and were not
4   comparable to the animals sacrificed at 76 weeks. At 76 weeks 3 of 10 the untreated and two of
5   the 10 corn oil treated controls were reported to have one small hepatocellular adenoma. None
6   of the controls examined at 76 weeks were reported to have any observed hepatocellular
7   carcinomas. The authors reported no cytotoxicity for TCE, corn oil, and untreated control group.
8   At 76 weeks, 75 mice treated with 800 mg/kg/day TCE were reported to have a prevalence of
9   50/75 animals having adenomas with the mean number of adenomas per animal to be 1.27 ± 0.14
10 (SEM). The prevalence of carcinomas in these same animals was reported to be 30/70 with the mean
11 number of hepatocellular carcinomas per animal to be 0.57 ± 0.10 (SEM). Although not comparable in
12 terms of time till tumor observation, Corn oil control animals examined at much later time points did
13 not have as great a tumor response as did those exposed to TCE. At 76-134 weeks 32 mice
14 treated with corn oil were reported to have a prevalence of 4/32 animals having adenomas with
15 the mean number of adenomas per animal to be 0.13 ± 0.06 (SEM). The prevalence of carcinomas in
16 these same animals was reported to be 4/32 with the mean number of hepatocellular carcinomas per
17 animal to be 0.12 ± 0.06 (SEM). Despite only examining one exposure level of TCE and the
18 limited reporting of findings other than incidence data, this study also reported that TCE
19 exposure in male B6C3F1 mice to be associated with increased induction of adenomas and
20 hepatocellular carcinoma, without concurrent cytotoxicity.
21
22   In terms of liver tumor phenotype, Anna et al. reported the % of H-ras codon 61
23 mutations in tumors from concurrent control animals (water and corn oil treatment groups
24 combined) examined in their study, historical controls in B6C3 F1mice, and in tumors from TCE
25 or DCA (0.5% in drinking water) treated animals. From their concurrent controls they reported
26 that H-ras codon 61 mutations in 17% (n=6) of adenomas and 100% (n=5) of carcinomas. For
27 historical controls (published and unpublished) they reported mutations in 73% (n=33) of
28 adenomas and mutations in 70% (n=30) of carcinomas.   For tumors from TCE treated animals
29 they reported mutations in 35% (n=40) of adenomas and 69% (n=36) of carcinomas, while for
30 DCA treated animals they reported mutations in 54% (n=24) of adenomas and in 68% (n=40) of
31 carcinomas. The authors reported that "in this study, the H-ras codon 61 mutation frequency was
32 not statistically different in liver tumors from dichloroacetic acid and trichloroethylene-treated
33 mice and combined controls (62%, 51% and 69%, respectively). " In regard to mutation spectra
34 in H-ras oncogenes detected B6C3F1 mouse liver "tumors," the authors reported combined
35 results for concurrent and historical controls of 58% AAA, 27% CGA, and 14% CTA
36 substitutions for CAA at Codon 61 out of 58 mutations. For TCE "tumors" the substitution
37 pattern was reported to be 29% AAA, 24% CGA, and 40% CTA substitutions for CAA at Codon
38 61 out of 39 mutations and for DCA 28% AAA, 35% CGA, and 38% CTA substitutions for
39 CAA at Codon 61 out of 40 mutations.
40
41 **2.2.22. Bull et al. 2002**
42
43   This study primarily presented results from exposures to TCE, DCA, TCA and

1   combinations of DCA and TCA after 52 weeks of exposure with some animals examined at 87
2   weeks. It only examined and described results for liver. In a third experiment, 1000 mg/kg TCE
3   was administered once daily 7 days a week for 79 weeks in 5% alkamuls in distilled water to 40
4   B6C3F1 male mice (6 weeks old at the beginning of the experiment). At the time of euthanasia,
5   the livers were removed, tumors identified, and the tissues section of for examination by a
6   pathologist and immunostaining. Liver weights were not reported. For the TCE gavage
7   experiment there were 6 gavage-associated deaths during the course of this experiment among a
8   total of 10 animals that died with TCE treatment. No animals were lost in the control group.
9   The limitations of this experiment were discussed in Caldwell et al. (2008b). Specifically, for
10 the DCA and TCA exposed animals, the experiment was limited by low statistical power, a
11 relatively short duration of exposure, and uncertainty in reports of lesion prevalence and
12 multiplicity due to inappropriate lesions grouping (i.e., grouping of hyperplastic nodules,
13 adenomas, and carcinomas together as "tumors"), and incomplete histopatholology
14 determinations (i.e., random selection of gross lesions for histopathology examination). For the
15 reported TCE results, Bull et al., (2002) reported a high prevalence (23/36 B6C3F1 male mice)
16 of adenomas and hepatocellular carcinoma (7/36) and gave results of an examination of
17 approximately half of the lesions induced by TCE exposure. Tumor incidence data was provided
18 for only 15 control mice and reported as 2/15 (13%) having adenomas and 1/15 (7%)
19 carcinomas. Thus, this study presents results that are consistent with other studies of chronic
20 exposure that show TCE induction of hepatocellular carcinoma in male B6C3F1 mice.
21
22   For determinations of immunoreactivity to c-Jun as a marker of differences in "tumor"
23 phenotype, Bull et al. (2002) did include all lesions in most of their treatment groups, decreasing
24 the uncertainty of his findings. The exceptions were the absence of control lesions and inclusion
25 of only 16/27 and 38/72 lesions for 0.5g/L DCA + 0.05 g/L TCA and 1g/kg/day TCE exposure
26 groups, respectively. Immunoreactivity results were reported for the group of hyperplastic
27 nodules, adenomas, and carcinomas. Thus, changes in c-Jun expression between the differing
28 types of lesions were not determined. Bull et al. (2002) reported lesion reactivity to c-Jun
29 antibody to be dependent on the proportion of the DCA and TCA administered after 52 weeks of
30 exposure. Given alone, DCA produced lesions in mouse liver for which approximately half
31 displayed a diffuse immunoreactivity to a c-Jun antibody, half did not, and none exhibited a
32 mixture of the two. After TCA exposure alone, no lesions were reported to be stained with this
33 antibody. When given in various combinations, DCA and TCA co-exposure induced a few
34 lesions that were only c-Jun+, many that were only c-Jun-, and a number with a mixed phenotype
35 whose frequency increased with the dose of DCA. For TCE exposure of 79 weeks, TCE-induced
36 lesions also had a mixture of phenotypes (42% c-Jun+, 34% c-Jun-, and 24% mixed and to be
37 most consistent with those resulting from DCA and TCA co-exposure but not either metabolite
38 alone.
39
40   Mutation frequency spectra for the H-ras codon 61 in mouse liver "tumors" induced by
41 TCE (n= 37 tumors examined) were reported to be significantly different than that for TCA (n=
42 41 tumors examined), with DCA-treated mice tumors giving an intermediate result (n= 64
43 tumors examined). In this experiment, TCA-induced "tumors" were reported to have more

1 mutations in codon 61(44%) than those from TCE (21%) and DCA (33%). This frequency of
2 mutation in the H-ras codon 61 for TCA is the opposite pattern as that observed for a number of
3 peroxisome proliferators in which the mutation spectra in tumors has been reported to be much
4 lower than spontaneously arising tumors (see Section 3.4.1.5.).   Bull et al. (2002) noted that the
5 mutation frequency for all TCE,TCA or DCA was lower in this experiment than for spontaneous
6 tumors reported in other studies (they had too few spontaneous tumors to analyze in this study),
7 but that this study utilized lower doses and was of shorter duration than that of Ferreira-Gonzalez
8 (1995). These are additional concerns along with the effects of lesion grouping in which a lower
9 stage of progression is group with more advanced stages.   In a limited subset of tumor that were
10 both sequenced and characterized histologically, only 8 of 34 (24%) TCE-induced adenomas but
11 9/15 (60%) of TCE-induced carcinomas had mutated H-ras at codon 61, which the authors
12 suggest is evidence that this mutation is a late event.
13
14   The issues involving identification of MOA through tumor phenotype analysis are
15 discussed in detail below for the more general case of liver cancer as well as for specific
16 hypothesized MOAs (see Sections 3.1.4., 3.1.8., 3.2.1., and 3.4.1.5). In an earlier paper, Bull
17 (2000) suggested that "the report by Anna et al (1994) indicated that TCE-induced tumors
18 possessed a different mutation spectra in codon 61 of the H-ras oncogene than those observed in
19 spontaneous tumors of control mice." Bull (2000) stated that "results of this type have been
20 interpreted as suggesting that a chemical is acting by a mutagenic mechanism" but went on to
21 suggest that it is not possible to a priori rule out a role for selection in this process and that
22 differences in mutation frequency and spectra in this gene provide some insight into the relative
23 contribution of different metabolites to TCE-induced liver tumors. Bull (2000) noted that data
24 from Anna et al. (1994), Ferreira-Gonzalez et al (1995), and Maronpot et al (1995) indicated that
25 mutation frequency in DCA-induced tumors did not differ significantly from that observed in
26 spontaneous tumors, that the mutation spectra found in DCA-induced tumors has a striking
27 similarity to that observed in TCE-induced tumors, and DCA-induced tumors were significantly
28 different than that of TCA-induced liver tumors. What is clear from these observations is the
29 phenotype of TCE-induced tumors appears to be more like DCA-induced tumors (which are
30 consistent with spontaneous tumors), or those resulting from a co-exposure to both DCA and
31 TCA, than from those induced by TCA. More importantly, these data suggest that using
32 measures other than dysplasticity and tincture indicate that mouse liver tumors induced by TCE
33 are heterogeneous in phenotype. The descriptions of tumors in mice reported by the NTP and
34 Maltoni et al studies are also consistent with phenotypic heterogeneity as well as consistency
35 with spontaneous tumor morphology.
36

2.3. Mode of Action: Relative Contribution of TCE Metabolites

38
39   Several metabolites of TCE have also been shown to induce liver cancer in rodents with
40 DCA and TCA having been the focus of study as potential active agent(s) of TCE liver toxicity
41 and/or carcinogenesis and both able to induce peroxisome proliferation (Caldwell and Keshava
42 2006). A variety of DCA effects from exposure have been noted that are consistent with
43 conditions that increase risk of liver cancer (e.g. effects on the cytosolic enzyme glutathione

1 (GST)-S-transferase-zeta, diabetes, and glycogen storage disease), with the pathological changes
2 induced by DCA on whole liver consistent with changes observed in preneoplastic foci from a
3 variety of agents (Caldwell and Keshava, 2006). Chloral hydrate is one of the first metabolites
4 from oxidative metabolism of TCE with a large fraction of TCE metabolism appearing to go
5 through chloral hydrate and then subsequent metabolism to TCA and trichloroethanol (Chiu et
6 al., 2006). Similarities in toxicity may indicate that common downstream metabolites may be
7 toxicologically important, and differences may indicate the importance of other metabolic
8 pathways.
9
10   Although both induce liver tumors, DCA and TCA have distinctly different actions
11 (Keshava and Caldwell, 2006) and apparently differ in tumor phenotype (see discussion above in
12 Section 2.2.8) and many studies have been conducted to try to elucidate the nature of those
13 differences (Caldwell et al 2007b). Limitations of all of the available chronic studies of TCA
14 and most of the studies of DCA include less than lifetime exposures, varying and small numbers
15 of animals examined, and few exposure concentrations that were relatively high.
16

2.3.1 Acute studies of DCA/TCA

18
19   The studies in this section focus on studies of DCA and TCA that examine, to the extent
20 possible, similar endpoints using similar experimental designs as those of TCE examined above
21 and that give insight into proposed MOAs for all three. Of note for any experiment involving
22 TCA, is whether exposure solutions were neutralized. Unbuffered TCA is commonly used as a
23 reagent to precipitate proteins so that any result from studies using unbuffered TCA could
24 potentially be confounded by the effects on pH.
25
26 *2.3.1.1. Sanchez and Bull 1990*
27
28   In this report TCA and DCA were administered to male B6C3F1 mice (9 weeks of age)
29 and male and female Swiss-Webster mice (9 weeks of age) for up to 14 days. At 2, 4, or 14 days,
30 mice were injected with tritiated thymidine. Experiments were replicated at least once but
31 results were pooled so that variation between experiments could not be determined. B6C3F1
32 male mice were given DCA or TCA at 0, 0.3 g/L, 1.0 g/L, 2.0 g/L in drinking water (n= 4 for
33 each group for 2 and 5 days, but n= 15 for control and n= 12 for treatment groups at day 14).
34 Swiss-Webster mice (N= 4) at were exposed to DCA only on Day 14 at 0, 1.0 or 2.0 g/L. Mice
35 were injected with tritiated thymidine 2 hours prior to sacrifice. The pH of the drinking water
36 was adjusted to 6.8 – 7.2 with sodium hydroxide. Concentrations of TCA and DCA were
37 reported to be stable for a minimum of 3 weeks. Hepatocyte diameters were reported to be
38 determined by randomly selecting 5 different high power fields (400X) in five different sections
39 per animals (total of 25 fields/ animal with "cells in and around areas of necrosis, close to the
40 edges of the section, or displaying mitotic figures were not included in the cell diameter
41 measurements." PAS staining was reported to be done for glycogen and lipofuscin determined
42 by autofluorescence. Tritiated thymidine was reported to be given to the animals 2 hours prior to

1 sacrifice. In 2 of 3 replications of the 14-day experiment, a portion of the liver was reported to
2 be set aside for DNA extraction with the remaining group examined autoradiographically for
3 tritiated thymidine incorporation into individual hepatocytes. Autoradiographs were also
4 reported to be examined in the highest dose of either DCA or TCA for the 2- and 5-day treatment
5 groups. Autoradiographs were reported to be analyzed in randomly selected fields (5 sections
6 per animal in 10 different fields) for a total of 50 fields/animal and reported as percentage of
7 cells in the fields which were labeled. There was no indication by the authors that they
8 characterized differing zones of the liver for preferential labeling. DNA thymidine incorporation
9 results were not examined in the same animals as those for individual hepatoctye incorporation
10 and also not examined at 2- or 5- day time periods. The only analyses reported for the Swiss-
11 Webster mice were of hepatic weight change and histopathology. Variations in results were
12 reported as standard error of the mean.
13
14   Liver weights were reported but not body weights so the relationship of liver/body weight
15 ratio could not be determined for the B6C3F1 mice. For liver weight, the numbers of animals
16 examined varied greatly between and within treatment groups. The number of control animals
17 examined were reported to be n = 4 on day 2, n=8 on day 5 and n=15 on day 14. There was also
18 a large variation between control groups in regard to liver weight. Control liver weights for day
19 2 were reported to be 1.3 ± 0.1, day 5 to be 1.5 ± 0.05 and for day 14 to be 1.3 ± 0.04 grams.
20 Liver weights in Day 5 control animals were much greater than those for day 2 and day 14
21 animals and thus the means varied by as much as 15%. For DCA, there was no reported change
22 in liver weights compared to controls values at any exposure level of DCA after 2 days of
23 exposure. After 5 days of exposure there was no difference in liver weight between controls and
24 0.3 g/l exposed animals. However the animals exposed at 1.0 or 2.0 g/l DCA had identical
25 increases in liver weight of 1.7 ± 0.13 and 1.7 ± 0.8 g, respectively. Due to the low power of the
26 experiment, only the 2.0 g/l DCA result was identified by the authors as significantly different
27 from the control value. For TCA there was a slight decrease reported between control values and
28 the 0.3 g/l treatment group (1.2 ± 0.1g vs. 1.3 ± 0.1) but the 1.0 g/l and 2.0 g/l treatment groups
29 had similar slight increases over control (for 1.0 g/l liver weight was 1.5 ± 0.1 and for 2.0 g/l
30 liver weight was 1.4 ± 0.1 g). The same pattern was apparent for the 5-day treatment groups for
31 TCA as for the 2-day treatment groups.
32
33   For 14 days exposure periods the number of animals studied was increased to12 for the
34 TCA and DCA treatment groups.   After 14 days of DCA treatment, there was a reported dose-
35 related increase in liver weight that was statistically significant at the two highest doses (i.e., at
36 0.3 g/l DCA liver weight was 1.4 ± 0.04, at 1.0 g/l DCA liver weight was 1.7 ± 0.07 g, and at 2.0
37 g/l DCA liver weight was 2.1 ± 0.08 g). This was 1.08-fold, 1.31-fold, and 1.62-fold of controls,
38 respectively. After 14 days of TCA exposure there was a dose-related increase in liver weight
39 that the authors reported to be statistically significant at all exposure levels (i.e., at 0.3 g/l liver
40 weight was 1.5 ± 0.06, at 1.0 g/l liver weight was 1.6 ± 0.07 g, and at 2.0 g/l liver weight was 1.8
41 ± 0.10 g). This represents 1.15-fold, 1.23-fold, and 1.38-fold of control. The authors note that at
42 14 days that DCA-associated increases in hepatic liver weight were greater than that of TCA.
43 What is apparent from this data is that while the magnitude of difference between the exposures

1   was ~ 6.7-fold between the lowest and highest dose, the differences between TCA exposure
2   groups for change in liver weight was ~ 2.5. For DCA the slope of the dose-response curve for
3   liver weight increases appeared to be closer to the magnitude of difference in exposure
4   concentrations between the groups (i.e. a difference of 7.7-fold between the highest and lowest
5   dose for liver weight induction). Given that the control animal weights varied as much as 15%,
6   the small number of animals examined, and that body weights were also not reported, there are
7   limitations for making quantitative comparisons between TCA and DCA treatments. However,
8   after 14 days of treatment it is apparent that there was a dose-related increase in liver weight
9   after either DCA or TCA exposure at these exposure levels. For male and female Swiss-Webster
10 mice 1 g/l and 2 g/l DCA treatment (n= 4) was reported to also induce an increase in %
11 liver/body weight that was similar to the magnitude of exposure difference (see below).
12
13   Grossly, livers of B6C3F1 mice treated with DCA for 1 or 2g/L were reported to have
14 "pale streaks running on the surface" and occasionally, discrete, white, round areas were also
15 observed on the surface of these livers. Such areas were not observed in TCA-treated or control
16 B6C3F1 mice." Swiss-Webster mice were reported to have "dose-related increases in hepatic
17 weight and hepatic/body weight ratios were observed. DCA-associated increases in relative
18 hepatic weights in both sexes were comparable to those in B6C3F1 mice. Pale streaks on the
19 surface of the liver were not observed in Swiss-Webster mice. Again there was no significant
20 effect on total body or renal weights (data not shown)." The authors report liver weights for the
21 Swiss-Webster male mice (n= 4 for each group) to be 2.1 ± 0.1 g for controls, 2.1 ± 0.1 g for 1.0
22 g/l DCA and 2.4 ± 0.2 g for 2.0 g/l DCA 14-day treatment groups. The % liver/body weights for
23 these same groups were reported to be 6.4 ± 0.4 %, 6.9 ± 0.2 %, and 8.1 ± 0.3 %, respectively.
24 For female Swiss-Webster mice (n= 4 for each group) the liver weights were reported to be 1.1 ±
25 0.1 g for controls, 1.5 ± 0.1 g for 1.0 g/l DCA and 1.7 ± 0.2 g for 2.0 g/l DCA 14-day treatment
26 groups. The % liver/body weights for these same groups of Swiss mice were reported to be 4.8 ±
27 0.2 %, 6.0 ± 0.2 %, and 6.8 ± 0.4 %, respectively. Thus, while there was no significant
28 difference in "liver weight" between the control and the 1.0 g/l DCA treatment group for male or
29 female Swiss-Webster mice, there was a statistically significant difference in liver/body weight
30 ratio reported by the authors. These data, illustrate the importance of reporting both measures
31 and the limitations of using small numbers of animals (n= 4 for the Swiss Webster vs. n= 12-14
32 for B6C3F1 14-days experiments). Relative liver weights were reported by the authors for male
33 B6C3F1 mice only for the 14-day groups, as a function of calculated mean water consumption,
34 as pooled data from the three experiments, and as a figure that was not comparable to the data
35 reported for Swiss-Webster mice. The liver weight data indicate that male mice of the same age
36 appeared to differ in liver weight between the two strains without treatment (i.e., male B6C3F1
37 mice had control liver weights at 14 days of 1.3 ± 0.04 g for 15 mice, while Swiss-Webster mice
38 had control values of 2.1 ± 0.1 for 4 mice). While the authors report that results were
39 "comparable" between the B6C3F1 mice in regard to DCA-induced changes in liver weight, the
40 increase in % liver/body weight ratios were 1.27-fold of control for Swiss-Webster male mice
41 (n= 4) and 1.42-fold fold of control for female while the increase in liver weight for B6C3F1
42 male mice (n= 12-14) was 1.62-fold of controls after 14 days of exposure to 2 g/l DCA.
43

1   The concentration of DNA in the liver was reported as mg hepatic DNA/g of liver. This
2   measurement can be associated with hepatocellular hypertrophy when decreased, or increased
3   cellularity (of any cell type), increased DNA synthesis, and/or increased hepatocellular ploidy in
4   the liver when increased. The number of animals examined for this parameter varied. For
5   control animals there were 4 animals reported to be examined at 2 days, 8 animals examined at 5
6   days, and at 14 days 8 animals were examined. The mean DNA content in control livers were
7   not reported to vary greatly however and the variation between animals was relatively low in the
8   5- and 14-day control groups (i.e., 1.67 ± 0.27 mg DNA/g, 1.70 ± 0.05 mgDNA/g, and 1.69 mg
9   DNA/g, for 2 -, 5-, or 14-day control animals, respectively). For treatment groups the number of
10 animals reported to be examined appeared to be the same as the control animals. For DCA
11 treatment there did not appear to be a dose-response in hepatic DNA content with the 1 g/l
12 exposure level having the same reported value as control but the 0.3 g/l and 2.0 g/l values
13 reported to be lower (mean values of 1.49 and 1.32 mg DNA/g, respectively). After 5 days of
14 exposure, all treatment groups were reported to have a lower DNA content that the control value
15 (i.e., 1.44 ± 0.06 mg DNA/g, $1.47 \pm$ mg DNA/g, and 1.30 ± 0.14 mg DNA/g, for 0.3 g/l, 1.0 g/l,
16 and 2.0 g/l exposure levels of DCA, respectively). After 14 days of exposure, there was a
17 reported increase in hepatic DNA at the 0.3 g/l exposure level but significant decreases at the 1.0
18 g/l and 2.0 g/l exposure levels (i.e., 1.94 ± 0.20 mg DNA/g, 1.44 ± 0.14 mg DNA/g, and $1.19 \pm$
19 0.16 mg DNA/g for the 0.3 g/l, 1.0 g/l, and 2.0 g/l exposure levels of DCA, respectively).
20 Changes in DNA concentration in the liver were not correlated with the pattern of liver weight
21 increases after DCA treatment. For example, while there was a clear dose-related increase in
22 liver weight after 14 days of DCA treatment, the 0.3 g/l DCA exposed group was reported to
23 have a higher rather than lower level of hepatic DNA than controls. After 2 or 5 days of DCA
24 treatment, liver weights were reported to be the same between the 1.0 g/l and 2.0 g/l treatment
25 groups but hepatic DNA was reported to be decreased.
26
27   For TCA there appeared to be a dose-related decrease in reported hepatic DNA after 2
28 days of treatment (i.e., 1.63 ± 0.07 mg DNA/g, 1.53 ± 0.08 mg DNA/g, and 1.43 ± 0.04 mg
29 DNA/g for the 0.3 g/l, 1.0 g/l, and 2.0 g/l exposure levels of TCA, respectively). After 5 days of
30 TCA exposure there was a reported decrease in hepatic DNA for all treatment groups that was
31 similar at the 1.0 g/l and 2.0 g/l exposure groups (i.e., 1.45 ± 0.17 mg DNA/g, 1.29 ± 0.18 mg
32 DNA/g, and 1.26 ± 0.22 mg DNA/g for the 0.3 g/l, 1.0 g/l, and 2.0 g/l exposure levels of TCA,
33 respectively). After 14 days of TCA treatment, there was a reported decrease in all treatment
34 groups in hepatic DNA content that did not appear to be dose-related (i.e., 1.31 ± 0.17 mg
35 DNA/g, 1.21 ± 0.17 mg DNA/g, and 1.33 ± 0.18 mg DNA/g for the 0.3 g/l, 1.0 g/l, and 2.0 g/l
36 exposure levels of TCA, respectively). Thus, similar to the results reported for DCA, the
37 patterns of liver weight gain did not match those of hepatic DNA decrease for TCA treated
38 animals. For example, although there appeared to be a dose-related increase in liver weight gain
39 after 14 days of TCA exposure, there was a treatment but not dose-related decrease in hepatic
40 DNA content.
41
42   In regard to the ability to detect changes, the low number of animals examined after 2
43 days of exposure (n = 4) limited the ability to detect a significant change in liver weight and

1   hepatic DNA concentration. For hepatic DNA determinations, the larger number of animals
2   examined at 5 and 14 day time points and the similarity of values with relatively smaller standard
3   error of the mean reported in the control animals made quantitative differences in this parameter
4   easier to determine.   However, animals varied in their response to treatment and this variability
5   exceeded that of the control groups. For DCA results reported at 14 days and those for TCA
6   reported at 5 and 14 days, the standard errors for treated animals showed a much greater
7   variability than those of the control animals (range of 0.04 – 0.05 mg DNA/g for control groups,
8   but ranges of 0.17 to 0.22 mg DNA/g for TCA at 5 days and 0.14 to 0.20 mg DNA/g for DCA or
9   TCA at 14 days).   The authors stated that "the increases in hepatic weights were generally
10   accompanied by decreases in the concentration of DNA. However, the only clear changes were
11   in animals treated with DCA for 5 or 14 days where the ANOVAs were clearly significant
12   ($P<0.020$ and 0.005, respectively). While changes of similar magnitude were observed in other
13   groups, the much greater variation observed in the treated groups resulted in not significant
14   differences by ANOVA ($p = 0.41$, 0.66. 0.26, 0.15 for DCA – 2 days, and TCA for 2,5, and 14
15   days, respectively)."

16
17   The size of hepatocytes is heterogeneous and correlated with its ploidy, zone, and age of
18   the animal (see Section 1.1 above). The authors do not indicate if there was predominance in
19   zone or ploidy for hepatocytes included in their analysis of average hepatocyte diameter in the
20   random selection of 25 fields per animal (n=3 to 7 animals). There appeared to be a dose-related
21   increase in cell diameter associated with DCA exposure and a treatment but not dose-related
22   increase with TCA treatment after 14 days of treatment. For control B6C3F1 male mice (n=7)
23   the hepatocyte diameter was reported to be 20.6 ± 0.4 microns. For mice exposed to DCA
24   hepatocyte diameter was reported to be 22.2 ± 0.2, 25.2 ± 0.6, and 26.0 ± 1.0 microns for 0.3 g/l,
25   1.0 g/l, and 2.0 g/l treated mice (n = 4 for each group), respectively. For mice exposed to TCA
26   hepatocyte diameter was reported to be 22.2 ± 0.2, 22.4 ± 0.6, and 23.2 ± 0.4 microns for 0.3 g/l,
27   1.0 g/l, and 2.0 g/l treated mice (n = 4 for the 0.3 g/l and 1.0 g/l groups and n= 3 for the 2.0 g/l
28   group), respectively. The small number of animals examined limited the power of the
29   experiment to determine statistically significant differences with the authors reporting that only
30   the 1.0 g/l DCA and 2.0 g/l DCA and TCA treated groups statistically significant from control
31   values. The dose-related increases in reported cell diameter were consistent with the dose-
32   related increases in liver weight reported for DCA after 14 days of exposure. However, the
33   pattern for hepatic DNA content did not. For TCA, the dose-related increases in cell diameter
34   were also consistent with the dose-related increases in liver weight after 14 days of exposure.
35   Similar to DCA results, the changes in hepatic DNA content did not correlate with changes in
36   cell size. In regard to the magnitude of increases over control values, the 68% vs. 38% increase
37   in liver weight for DCA vs. TCA at 2.0 g/l, was less than the 26% and 13% increases in cell
38   diameter for the same groups, respectively. Therefore, for both DCA and TCA exposure there
39   appeared to be dose-related hepatomegaly and increased cell size after 14-days of exposure.
40
41   The authors reported PAS staining for glycogen content as an attempt to examine the
42   nature of increased cell size by DCA and TCA. However, they did not present any quantitative
43   data and only provided a brief discussion. The authors reported that "hepatic sections of DCA-

1   treated B6C3F1 mice (1 and 2 g/l) contained very large amounts of perilobular PAS-positive
2   material within hepatocytes. PAS stained hepatic sections from animals receiving the highest
3   concentration of TCA displayed a much less intense staining that was confined to periportal
4   areas. Amylase digesting confirmed the majority of the PAS-positive material to by glycogen.
5   Thus, increased hepatocellular size in groups receiving DCA appears to be related to increased
6   glycogen deposition. Similar increases in glycogen deposition were observed in Swiss-Webster
7   mice." There is no way to discern whether DCA-induced glycogen deposition was dose-related
8   and therefore correlated with increased liver weight and cell diameter. While the authors suggest
9   that Swiss-Webster mice displayed "similar increased in glycogen deposition" the authors did
10   not report a similar increase in liver weight gain after DCA exposure at 14 days (1.27-fold of
11   control % liver/body weight ratio in Swiss male mice and 1.42-fold in female Swiss-Webster
12   mice vs. 1.62-fold of control in B6C3F1 mice after 14 days of exposure to 2 g/l DCA). Thus, the
13   contribution of glycogen deposition to DCA-induced hepatomegaly and the nature of increased
14   cell size induced by acute TCA exposure cannot be determined by this study. However, this
15   study does show that DCA and TCA differ in respect to their effects on glycogen deposition after
16   short-term exposure and the data suggest that .
17

18   The authors report that "localized areas of coagulative necrosis were observed
19   histologically in both B6C3F1 and Swiss-Webster mice treated with DCA at concentrations of 1
20   and 2 g/l for 14 days. The necrotic areas corresponded to the pale streaked areas seen grossly.
21   These areas varied in size, shape and location within sections and occupied up to several mm^2.
22   An acute inflammatory response characterized by thin rims of neutrophils was associated with
23   the necrosis, along with multiple mitotic figures. No such areas of necrosis were observed in
24   animals treated at lower concentrations of DCA, or in animals receiving the chemical for 2 or 5
25   days. Mice treated with 2 g/l TCA for 14 days have some necrotic areas, but at such low
26   frequency that it was not possible to determine if it was treatment-related (2 lesions in a total of
27   20 sections examined). No necrosis was observed in animals treated at the lower concentrations
28   of TCA or at earlier time points." Again there were no quantitative estimates given of the size
29   of necrotic areas, variation between animals, variation between strain, or dose-response of
30   necrosis reported for DCA exposure by the authors. The lack of necrosis after 2 and 5 days of
31   exposure at all treatment levels and at the lower exposure level at 14 days of exposure is not
32   correlated with the increases in liver weight reported for these treatment groups.
33

34   Autoradiographs of randomly chosen high powered fields (400X) (50 fields/animal) were
35   reported as the percentage of cells in the fields that were labeled. There was significant variation
36   in the number of animals examined and in the reported mean % of labeled cells between control
37   groups. The number of control animals was not given for the 2-day group but for the 5-day and
38   14 day groups were reported to be n = 4 and n = 11, respectively. The mean % of labeling in
39   control animals was reported at 0.11 ± 0.03, 0.12 ± 0.04, and $0.46 \pm 0.07\%$ of hepatocytes for 2-
40   day, 5-day, and 14-day control groups, respectively. Only the 2.0 g/l exposures of DCA and
41   TCA were examined at all 3 times of exposure while all groups were examined at 14 days.
42   However, the number of animals examined in all treatment groups appeared to be only 4 animals
43   in each group. There was not an increase over controls reported in the 2.0 g/l DCA or TCA 2-

1 and 5-day exposure groups in hepatocyte labeling with tritiated thymidine. After 14 days of
2 exposure, there was a statistically significant but very small dose-related increase over the
3 control value after DCA exposure (i.e., 0.46 ± 0.07, 0.64 ± 0.15, 0.75 ± 0.22, and 0.94 ± 0.05 %
4 labeling of hepatocytes in control, 0.3 g/l, 1.0 g/l and 2.0 g/l DCA treatment groups,
5 respectively). For TCA, there was no change in hepatocyte labeling except for a 50% decrease
6 from control values at after 14 days of exposure to 2.0 g/l TCA (i.e., 0.46 ± 0.07, 0.50 ± 0.14,
7 0.52 ± 0.26, and 0.26 ± 0.14 % labeling of hepatocytes in control, 0.3 g/l, 1.0 g/l and 2.0 g/l TCA
8 treatment groups, respectively). The authors report that "labeled cells were localized around
9 necrotic areas in these [sic DCA treated] groups. Since counts were made randomly, the local
10 increased in DCA-treated animals at concentrations of 1 and 2 g/l are in fact much higher than
11 indicated by the data. Labeling indices in these areas of proliferation were as high as 30%.
12 Labeled hepatocytes in TCA-treated and the control animals were distributed uniformly
13 throughout the sections. There was an apparent decrease in the percentage of labeled cells in the
14 group of animals treated with the highest dose of TCA. This is because no labeled cells were
15 found in any of the fields examined for one animal."   The data for control mice in this
16 experiment is consistent with others showing that the liver is quiescent in regard to
17 hepatocellular proliferation with few cells undergoing mitosis (see Section 1.1). For up to 14
18 days of exposure with either DCA or TCA, there is little increase in hepatocellular proliferation
19 except in instances and in close proximity to areas of proliferation. The increases in liver weight
20 reported for this study were not correlated with and cannot be a result of hepatocellular
21 proliferation as only a very small population of hepatocytes is undergoing DNA synthesis. For
22 TCA, there was no increase in DNA synthesis in hepatocytes, even at the highest dose, as shown
23 by autoradiographic data of tritiated thymidine incorporation in random fields.
24
25   Whole liver sections were examined for tritiated thymidine incorporation from DNA
26 extracts. The number of animals examined varied (i.e., n = 4 for the 2-day exposure groups and
27 n = 8 for 5- and 14-day exposure groups) but the number of control animals examined were the
28 same as the treated groups for this analysis. The levels of tritiated thymidine incorporation in
29 hepatic DNA (dpm/mg DNA expressed as mean x $10^3 \pm$ SE of N animals) were reported to be
30 similar across control groups (i.e., 56 ± 11, 56 ± 6, and 56 ± 7 dpm/mg DNA, for 2-day, 5-day,
31 and 14-day treatment groups, respectively).   After two days of DCA exposure, there appeared to
32 be a slight treatment-related but not dose-related increase in reported tritiated thymidine
33 incorporation into hepatic DNA (i.e., 72 ± 23, 80 ± 6, and 68 ± 7 dpm/mg DNA for 0.3, 1.0, or
34 2.0 g/l DCA, respectively). After 5 days of DCA exposure, there appeared to be a dose-related
35 increase in reported tritiated thymidine incorporation into hepatic DNA (i.e., 68 ± 18, 110 ± 20,
36 and 130 ± 7 dpm/mg DNA for 0.3, 1.0, or 2.0 g/l DCA, respectively). However, after 14 days of
37 DCA exposure, levels of tritiated thymidine incorporation were less than those reported at 5 days
38 and the level for the 0.3 g/l exposure group was less than the control value (i.e., 33 ± 11, 77 ± 9,
39 and 81 ± 12 dpm/mg DNA for 0.3, 1.0, or 2.0 g/l DCA, respectively). After two days of TCA
40 exposure there did not appear to be a treatment-related increase in tritiated thymidine
41 incorporation into hepatic DNA (i.e., 82 ± 16, 52 ± 7, and 54 ± 7 dpm/mg DNA for 0.3, 1.0, or
42 2.0 g/l TCA, respectively). Similar to the reported results for DCA, after 5 days of TCA
43 exposure there appeared to be a dose-related increase in reported tritiated thymidine

1   incorporation into hepatic DNA (i.e., 79 ± 23, 86 ± 17, and 158 ± 33 dpm/mg DNA for 0.3, 1.0,
2   or 2.0 g/l TCA, respectively). After 14 days of TCA exposure there were treatment related
3   increases but not a dose-related increase in reported tritiated thymidine incorporation into hepatic
4   DNA (i.e., 71 ± 10, 73 ± 14, and 103 ± 14 dpm/mg DNA for 0.3, 1.0, or 2.0 g/l TCA,
5   respectively). It would appear that for both TCA and DCA the increase in tritiated thymidine
6   incorporation into hepatic DNA was dose related and peaked after 5 days of exposure. The
7   authors report that the decrease in incorporation into hepatic DNA observed after 14 days of
8   DCA treatment at 0.3 g/l to be statistically significant as well as the increases after 5 and 14 days
9   of TCA exposure at the 2.0 g/l level. The small numbers of animals examined, the varying
10 number of animals examined, and the degree of variation in treatment-related effects limits the
11 statistical power of this experiment to detect quantitative changes.
12
13   Given the limitations of this experiment, determination of an accurate measure of the
14 quantitative differences in tritiated thymidine incorporation into whole liver DNA or that
15 observed in hepatocytes are hard to determine. In general the results for tritiated thymidine
16 incorporation into hepatic DNA were consistent with those for tritiated thymidine incorporation
17 into hepatocytes in that they show that there were at most a small population of hepatocytes
18 undergoing DNA synthesis after up to 14 days of exposure at relative high levels of exposure to
19 DCA and TCA (i.e., the largest percentage of hepatocytes undergoing DNA synthesis for any
20 treatment group was less than 1% of hepatocytes). The highest increases over control levels for
21 hepatic DNA incorporation for the whole liver were reported at the highest exposure level of
22 TCA treatment after 5 days of treatment (3-fold of control) and after 14 days of TCA treatment
23 (2-fold of control). Although the authors report small areas of focal necrosis with concurrent
24 localized increases in hepatocyte proliferation in DCA treated animals exposed to1.0 g/l and 2.0
25 g/l DCA, the levels of whole liver tritiated thymidine incorporation were only slightly elevated
26 over controls at these concentrations, and were decreased at the 0.3 g/l exposure concentration
27 for which no focal necrosis was reported. The whole liver DNA incorporation of tritiated
28 thymidine was not consistent with the pattern of tritiated thymidine incorporation observed in
29 individual hepatocytes. The authors state that "at present, the mechanisms for increased tritiated
30 thymidine uptake in the absence of increased rates of cell replication with increasing doses of
31 TCA cannot be determined." The authors do not discuss the possibility that the difference in
32 hepatocyte labeling and whole liver DNA tritiated thymidine incorporation could have been due
33 to the labeling representing increased polyploidization rather than cell proliferation, as well as
34 increased numbers of proliferating nonparenchymal and inflammatory cells. The increased cell
35 size due from TCA exposure without concurrent increased glycogen deposition could have been
36 indicative of increased polyploidization. Finally, although both TCA- and DCA-induced
37 increases in liver weight were generally consistent with cell size increases, they were not
38 correlated with patterns of change in hepatic DNA content, incorporation of tritiated thymidine
39 in DNA extracts from whole liver, or incorporation of tritiated thymidine in hepatocytes. In
40 regard to cell size, although increased glycogen deposition with DCA exposure was noted by the
41 authors of this study, lack of quantitative analyses of that accumulation precludes comparison
42 with DCA-induced liver weight gain.
43

1   *2.3.1.2. Nelson et al. 1989*
2
3   Nelson and Bull (1988) administered TCE (0, 3.9, 11.4, 22.9, and 30.4 mmol/kg) in
4   tween 80 via gavage to male Sprague Dawley rats and male B6C3F1 mice, sacrificed them fours
5   hours after treatment (n= 4-7), and measured the rate of DNA unwinding under alkaline
6   conditions. They assumed that this assay represented increases in single-strand breaks. For rats
7   there was little change from controls up to 11.4 mmol/kg (1.5. g/kg TCE) but a significantly
8   increased rate of unwinding at 22.9 and 30.4 mmol/kg TCE (~ 2-fold greater at 30.4 mmol). For
9   mice there was a significantly increased level of DNA unwinding at 11.4 and 22.9 mmol.
10   Concentrations above 22.9 mmol/kg were reported to be lethal to the mice. In this same study,
11   TCE metabolites were administered in unbuffered solution using the same assay. DCA was
12   reported to be most potent in this assay with TCA being the lowest, while chloral hydrate (CH)
13   closely approximated the dose-response curve of TCE in the rat. In the mouse the most potent
14   metabolite in the assay was reported to be TCA followed by DCA with CH considerably less
15   potent.
16
17   The focus of the Nelson et al. (1989) study was to examine whether reported single strand
18   breaks in hepatic DNA induced by DCA and TCA (Nelson and Bull, 1988) were secondary to
19   peroxisome proliferation also reported to be induced by both. Male B6C3F1 mice (25-30 g but
20   no age reported) were given DCA (10 mg/kg or 500 mg/kg) or TCA (500 mg/kg) via gavage in
21   1% aqueous tween 80 with no pH adjustment. The animals were reported to be sacrificed 1, 2, 4,
22   or 8 hours after administration and livers examined for single strand breaks as a whole liver
23   homogenate. In a separate experiment (experiment #2) treatment was parallel to the first (500
24   mg/kg treatment of DCA or TCA) but levels of PCO activity were measured as an indication of
25   peroxisome proliferation and expressed as μmol/min/g liver. In a separate experiment
26   (experiment #3) mice were administered 500 mg/kg DCA or TCA for 10 days with Clofibrate
27   administered at a dose of 250 mg/kg as a positive control. 24 hours after the last dose, animals
28   were killed and liver examined by light microscopy and PCO activity.   Finally, in an experiment
29   parallel in design to experiment #3, single strand breaks were measured in total hepatic DNA
30   after 500 mg/kg exposure to TCA (experiment #4). Electron microscopy was performed on 2
31   animals/group for vehicle, DCA or TCA treatment, with 6 randomly chosen micrographic fields
32   utilized for peroxisome profiles. These micrographs were analyzed without identification as to
33   what area of the liver lobules they were being taken from. Hence there is a question as to
34   whether the areas which are known to be peroxisome rich were assayed of not.
35
36   The data from all control groups were reported as pooled data in figures but statistical
37   comparisons were made between concurrent control and treated groups. The results for DNA
38   single strand breaks were reported for "13 control animals" and each experimental time point "as
39   at least 6 animals. DNA strand breaks were reported to be significantly increased over
40   concurrent control by a single exposure to 10 or 500 mg/kg DCA or 500 mg/kg TCA for 1, 2 or 4
41   hours after administration but not at 8 or 24 hours. There did not appear to be a difference in the
42   magnitude of response between the 3 treatments (the fraction of unwound DNA was ~ 2.5 times
43   that of control). PCO activity was reported to be not increased over control within 24 hours of

1   either DCA or TCA treatment. (n = 6 animals per group). The fraction of alkaline unwinding
2   rates as an indicator of single strand breaks were reported to not be significantly different from
3   controls and TCA-treated animals after 10 days of exposure (n= 5).
4
5   Relative to controls, body weights were reported to not be affected by exposures to DCA
6   or TCA for 10 days at 500 mg/kg (with data was not shown.) (n=6 per group).   However, both
7   DCA and TCA were reported to significantly increase liver weight and liver/body weight ratios
8   (i.e., liver weights were 1.3 ± 0.05 g, 2.1 ± 0.10 g, and 1.7 ± 0.09 g for control, 500 mg/kg DCA
9   and 500 mg/kg TCA treatment groups, respectively while % liver/body weights were 4.9 ± 0.14
10 %, 7.5 ± 0.18 %, and 5.7 ± 0.14 % for control, 500 mg/kg DCA and 500 mg/kg TCA treatment
11 groups, respectively).   PCO activity (μmol/min/g liver) was reported to be significantly
12 increased by DCA (500 mg/kg), TCA (500 mg/kg), and Clofibrate (250 mg/kg) treatment (i.e.,
13 levels of oxidation were 0.63 ± 0.07, 1.03 ± 0.09, 1.70 ± 0.08, and 3.26 ± 0.05 for control, 500
14 mg/kg DCA, 500 mg/kg TCA and 250 mg/kg Clofibrate treatment groups, respectively). Thus,
15 the increases were ~ 1.63-fold, 2.7 –fold, and 5-fold of control for DCA, TCA and Clofibrate
16 treatments. Results from randomly selected electron photomicrographs from 2 animals (6 per
17 animal) were reported for DCA and TCA treatment and to show an increase in peroxisomes per
18 unit area that was reported to be statistically significant (i.e., 9.8 ± 1.2, 25.4 ± 2.9, and 23.6 ± 1.8
19 for control, 500 mg/kg DCA and 500 mg/kg TCA, respectively). The 2.5-fold and 2.4-fold of
20 control values for DCA and TCA gave a different pattern than that of PCO activity. The small
21 number of animals examined limited the power of the experiment to quantitatively determine the
22 magnitude of peroxisome proliferation via electron microscopy. The enzyme analyses suggested
23 that both DCA and TCA were weaker inducers of peroxisome proliferation that Clofibrate.
24
25   The authors report that there was no evidence of gross hepatotoxicity in vehicle or TCA-
26 treated mice. Light microscopic sections from mice exposed to TCA or DCA for 10 days were
27 stained with H&E and PAS for glycogen. For TCA treatment, PAS staining "produced
28 approximately the same intensity of staining and amylase digesting revealed that the vast
29 majority of PAS-positive staining was glycogen." Hepatocytes were reported to be "slightly
30 larger in TCA-treated mice than hepatocytes from control animals throughout the liver section
31 with the architecture and tissue pattern of the liver intact. The histopathology after DCA
32 treatment was reported to be "markedly different than that observed with either vehicle or TCA
33 treatments" with the "most pronounced change in the size of hepatocytes." DCA was reported to
34 "produce marked cellular hypertrophy uniformly throughout the liver. The hepatocytes were
35 approximately 1.4 times larger in diameter than control liver cells. This hypertrophy was
36 accompanied by an increase in PAS staining; indicating greater glycogen deposition than in
37 TCA-treated and control liver tissue. Multiple white streaks were grossly visible on the surface
38 of the liver of DCA-treated mice. The white areas corresponded with subcapsular foci of
39 coagulative necrosis. These localized necrotic areas were not encapsulated and varied in size.
40 The largest necrotic foci occupied the area of a single lobule. These necrotic areas showed a
41 change in staining characteristics. Often this change consisted of increased eosinophilia. A
42 slight inflammatory response, characterized by neutrophil infiltration, was present. These
43 changed were evident in all DCA-treated mice."

1   The results from this experiment cannot inform as to dose-response relationships for the
2   parameters tested with the exception of DNA single strand breaks where 2 concentrations of
3   DCA were examined (10 mg/kg and 500 mg/kg). For this parameter the 10 mg/kg exposure of
4   DCA was as effective as the 500 mg/kg dose where toxicity was observed. This effect on DNA
5   was also observed before evidence of induction of peroxisome proliferation. The authors did not
6   examine Clofibrate for effects on DNA so whether it too, would have produced this effect is
7   unclear. The results from this study are consistent with those of Sanchez and Bull (1990) for
8   induction of hepatomegaly by DCA and TCA, the lack of hepatotoxicity at this dose by TCA,
9   and the difference in glycogen deposition between DCA and TCA.
10
11   *2.3.1.3. Styles et al. 1991*
12
13   In this report a similar paradigm is used as Nelson et al (1989) for the determination of
14   repeating that work on single strand breakage and to study DNA synthesis and peroxisome
15   proliferation. In regard to the findings of single strand breaks, Styles et al (1991) reported for a
16   similar paradigm of 500 mg/kg neutralized TCA administered to male B6C3F1 mice (7-8 weeks
17   of age) examined at 1, 4, 8 and 24 hours after dosing, reported no increased unwinding of DNA 1
18   or 24 hours after TCA administration. In a separate experiment tritiated thymidine was
19   administered to mice 1 hour before sacrifice at 24, 36, 48, 72, and 96 hours after the first dose of
20   500 mg/kg TCA for 3 days via gavage (n= 5 animals per group).
21
22   The hepatic DNA uptake of tritiated thymidine was reported to be similar to control
23   levels up to 36 hours after the first dose and then to increase to a level ~ 6 fold greater than
24   controls by 72 hours after the first dose of TCA. By 96 hours the level of tritiated thymidine
25   incorporation had fallen to ~ 4 –fold greater than controls. The variation, reported by S.D. was
26   very large in treated animals (e.g., S.D. was equal to ~ ± 1.3 fold of control for 48 hour time
27   point).   Individual hepatocytes were examined with the number of labeled hepatocytes /1000
28   cells reported for each animal. The control level was reported to be ~ 1 with a S.D. of similar
29   magnitude. The number of labeled hepatocytes was reported to decrease between 24 and 36
30   hours and then to rise slowly back to control levels at 48 hour and then to be significantly
31   increased 72 hours after the first dose of TCA (~ 9 cells/1000 with a S.D of 3.5) and then to
32   decrease to a level of ~ 5 cells/1000. Thus, it appears that increases in hepatic DNA tritiated
33   thymidine uptake preceded those of increased labeled hepatocytes and did not capture the
34   decrease in hepatocyte labeling at 36 hours. By either measure the population of cells
35   undergoing DNA synthesis was small with the peak level being less than 1% of the hepatocyte
36   population. The authors go on to report the zonal distribution of mean number of hepatocytes
37   incorporating tritiated thymidine but no variations between animals were reported. The decrease
38   in hepatocyte labeling at 36 hours was apparent at all zones. By 48 hours there appeared to be
39   slightly more perioportal than midzonal cells undergoing DNA synthesis with centrilobular cells
40   still below control levels. By 72 hours all zones of the liver were reported to have a similar
41   number of labeled cells. By 96 hours the midzonal and centrilobular regions have returned
42   almost to control levels while the periportal areas were still elevated. These results are consistent
43   with all hepatocytes showing a decrease in DNA synthesis by 36 hours and then a wave of DNA

1 synthesis occurring starting at the periportal zone and progressing through to the pericentral zone
2 until 72 hours and then the midzonal and pericentral hepatocytes completing their DNA
3 synthesis activity. Peroxisome proliferation was assessed via electron photomicrographs taken
4 in mice (4 controls and 4 treated animals) given 10 daily doses of 500 mg/kg TCA and killed 14
5 hours after the last dose. No details were given by the authors as to methodology for peroxisome
6 volume estimate (e.g., how many photos per animals were examined and whether they were
7 randomly chosen). The mean % cell volume occupied by peroxisome was reported to be 2.1 ±
8 0.386 and 3.9 ± 0.551 % for control and 500 mg/kg TCA, respectively. Given there were no
9 time points examined before 10 days for peroxisome proliferation, correlations with DNA
10 synthesis activity induced by TCA cannot be made from this experiment. However, it is clear
11 from this study that a wave of DNA synthesis occurs throughout the liver after treatment of TCA
12 at this exposure concentration and that it has peaked by 72 hours even with continuous exposure
13 to 96 hours. Whether the DNA synthesis represents polyploidization or cell proliferation cannot
14 be determined from this data as neither can a dose-response.
15
16 *2.3.1.4. Carter et al. 1995*
17
18   The aim of this study was to "use correlative biochemical, pathologic and morphometric
19 techniques to characterize and quantify the acute, short-term responses of hepatocytes in the
20 male B6C3F1 mouse to drinking water containing DCA." This report used tritiated thymidine
21 incorporation, DNA concentration, hepatocyte number per field (cellularity), nuclear size and
22 binuclearity (polyploidy) parameters to study 0, 0.5 and 5 g/l neutralized DCA exposures up to
23 30 days. Male B6C3F1 mice were started on treatment at 28 days of age. Tritiated thymidine
24 was administered by miniosmotic pump 5 days prior to sacrifice. The experiment was conduced
25 in two phases which consisted of 5-15 days of treatment (phase I) and 20-30 days of treatment
26 (phase II) with 5 animals per group in groups sacrificed at 5-day intervals. Liver sections were
27 stained for H&E, PAS (for glycogen) or methyl green pryonin stain (for RNA). DNA was
28 extracted from liver homogenates and the amount of tritiated thymidine determined as dpm/μg
29 DNA. Autoradiography was performed with the number of hepatocyte nuclei scored in 1000
30 hepatocytes selected randomly to provide a labeling index of "number of labeled cells/1000 X
31 100%". Changes in cellularity, nuclear size and number of multinucleate cells were quantified
32 in H&E sections at 40 X power. Hepatocyte cellularity was determined by counting the number
33 of nuclei in 50 microscopic fields with multinucleate cells being counted as one cell and
34 nonparenchymal cells not counted. Nuclear size was also measured in 200 nuclei with the mean
35 area plus 2 S.D. was considered to be the largest possible single nucleus. Therefore, polyploid
36 diploid cells were identified by the authors but not cells that had undergone polyploidy with
37 increased DNA content in a single nucleus.
38
39   Mean body weights at the beginning of the experiment varied between 18.7 and 19.6 g in
40 the first 3 exposure groups of phase I of the study. Through 15 days of exposure there did not
41 appear to be a change in body weight in the 0.5 g/l exposure groups but in the 5g/l exposure
42 group body weight was reduced at 5, 10 and 15 days with that reduction statistically significant
43 at 5 and. 15 days. Liver weights did not appear to be increased at day 5 but were increased at

1   days 10 and 15 in both treatment groups (i.e., means ± S.E.M. for day 10; 1.36 ± 0.03, 1.46 ±
2   0.03, and 1.59 ± 0.08 g for control, 0.5 g/l and 5 g/l DCA, respectively and for day 15; 1.51 ±
3   0.06, 1.72 ± 0.05, and 2.08 ± 0.11 g for control, 0.5 g/l and 5 g/l DCA, respectively). The %
4   liver/body weight followed a similar pattern with the exception that at day 5 the 5g/l exposure
5   group had a statistically significant increase over control (i.e., for day 10; 6.00 ± 0.10, 6.72 ±
6   0.17, and 8.21 ± 0.10 % for control, 0.5 g/l and 5 g/l DCA, respectively and for day 15; 6.22 ±
7   0.08, 6.99 ± 0.15, and 10.37 ± 0.27 % g for control, 0.5 g/l and 5 g/l DCA, respectively).
8
9   In phase II of the study, control body weights were smaller than phase I and varied
10   between 16.6 and 16.9 g in the first 3 exposure groups. Liver weights of controls were also
11   smaller making it difficult to quantitatively compare the two groups in terms of absolute liver
12   weights. However, the pattern of DCA-induced increases in liver weight and % liver/body
13   weight remained. The patterns of body weight reduction only in the 5g/l treatment groups and
14   increased liver weight with DCA treatment at both concentrations continued from 20 to 30 days
15   of exposure. For liver weight there was a slight but statistically significant increase in liver
16   weight for the 0.5 g/l treatment groups over controls (i.e., for day 20; 1.02 ± 0.02, 1.18 ± 0.05,
17   and 1.98 ± 0.05 g for control, 0.5 g/l and 5 g/l DCA, respectively, for day 25; 1.15 ± 0.03, 1.34 ±
18   0.04, and 2.06 ± 0.12 g for control, 0.5 g/l and 5 g/l DCA, respectively, for day 30; 1.15 ± 0.03,
19   1.39 ± 0.08, and 1.90 ± 0.12 g for control, 0.5 g/l and 5 g/l DCA, respectively). For % liver/body
20   weight there was a small increase at 0.5 g/l that was not statistically significant but all other
21   treatments induced increases in % liver/body weight that were statistically significant (i.e., for
22   day 20; 4.82 ± 0.07, 5.05 ± 0.09, and 9.71 ± 0.11 % for control, 0.5 g/l and 5 g/l DCA,
23   respectively, for day 25; 5.08 ± 0.04, 5.91 ± 0.09, and 10.38 ± 0.58 % for control, 0.5 g/l and 5
24   g/l DCA, respectively, for day 30; 5.17 ± 0.09, 6.01 ± 0.08, and 10.28 ± 0.28 % for control, 0.5
25   g/l and 5 g/l DCA, respectively). Of note is the dramatic decrease in water consumption in the 5
26   g/l treatment groups which were consistently reduced by 64% in phase I and 46% in phase II.
27   The 0.5g/l treatment groups had no difference from controls in water consumption at any time in
28   the study. The effects of such water consumption decreases would affect body weight as well as
29   dose received. Given the differences in the size of the animals at the beginning of the study and
30   the concurrent differences in liver weights and % liver/body weight in control animals between
31   the two phases, the changes in these parameters through time from DCA treatments cannot be
32   accurately determined (e.g., control liver/body weights averaged 6.32% in phase I but 5.02% in
33   phase II). However, % liver/body weight increase were reported to be consistently increased
34   within and between both phases of the study for the 0.5 g/l DCA treatment from 5 days of
35   treatment to 30 days of treatment (i.e., for phase I the average increase was 9.5% and for phase II
36   the average increased was 12.5% for 0.5 g/l DCA treated groups). Although increase at 5 days
37   the nonsignificance of the change may be resultant from the small number of animals examined.
38   The difference in magnitude of dose and % liver/body weight increase is difficult to determine
39   given that the 5 g/l dose of DCA reduced body weight and significantly reduced water
40   consumption by ~ 50% in both phases of the study. Of note is that the differences in DCA-
41   induced % liver/body weight were ~ 6-fold for the 15, 25, and 30-day data between the 0.5 and 5
42   g/l DCA exposures rather than the 10-fold difference in exposure concentration in the drinking
43   water.

The incorporation of tritiated thymidine into total hepatic DNA control treatment groups was reported to be 73.34 ± 11.74 dpm/µg DNA at 5 days, 34 ± 4.12 dpm/µg DNA at 15 days, and 28.48 ± 3.24 dpm/µg DNA at 20 days but was not reported for other treatments. The results for 0.5g/l treatments were not reported quantitatively but the authors stated that the results "showed similar trends of initial inhibition followed by enhancement of labeling, the changes relative to controls were not statistically significant." For 5 g/l treatment groups the 5-day treated groups DNA tritiated thymidine incorporation was reported to be 42.8% of controls and followed by a transient increase at 15 and 20 days (i.e., 2.65- and 2.45-fold of controls, respectively) but after 25 and 30 days to not be significantly different from controls (data not shown). Labeling indices of hepatocytes were reported as means but variations as either SEM or S.D. were not reported. Control means were reported as 5.5, 4, 2, 2, 3.2, and 3.5 % of randomly selected hepatocytes for 5, 10, 15, 20, 25, and 30 days, respectively, for 4 to 5 animals per group. In contrast to the DNA incorporation results, no increase in labeling of hepatocytes was reported to be observed in comparison to controls for any DCA treatment group from 5 to 30 days of DCA exposure. The 5 g/l treatment group showed an immediate decrease in hepatocyte labeling from day 5 onwards that gradually increased ~ half of control levels by day 30 of exposure (i.e., <0.5 % LI at day 5, ~ 1% LI at day 10, ~0.6% LI at day 20, 1% LI at day 25 and 2% LI at day 30). For the 0.5 g/l treatment the labeling index was reported to not differ from controls from days 5 though 15 but to be significantly decreased between days 20 and 30 to levels similar to those observed for the 5 g/l exposures. The relatively higher number of hepatocytes incorporating label reported in this study than others can be reflection of the longer times of exposure to tritiated thymidine. Here, incorporation was shown for 1 weeks worth of exposure and reflects the % of cell undergoing synthesis during that time period. Also the higher labeling index in control animals at the 5 and 10 day exposure periods is probably a reflection of the age of the animals at the time of study. From the data reported by the authors, there was a correlation between the patterns of total DNA incorporation of label and hepatocyte labeling indices in control groups (i.e. higher level of labeling at 5 days than at 15 and 20). However, the patterns of decreased thymidine labeling reported for hepatocytes were not correlated with a transient increase in total DNA thymidine incorporation reported with DCA treatment, especially at the 5 g/l exposure level with a large decrease reported for the number of labeled hepatocytes at the same time an increase in total DNA thymidine incorporation was reported. Although reported to be transiently increased, the total hepatic DNA labeling still represented at most a 2.5 fold increase over control liver which represents a small population of cells. Given that the study examined hepatocyte labeling in random fields and did not report quantitative zonal differences in proliferation, a more accurate determination of what hepatocytes were undergoing proliferation cannot be made from the LI results. Also although the authors report signs of inflammatory cells for 5-day treatment there is no reference to any inflammatory changes that may have been observed at later time periods when cellular degeneration and loss of nuclei were apparent. Such an increase inflammatory infiltrates can increase the DNA synthesis measurements in the liver. The difference in LI and total DNA synthesis could reflect differences in nonparenchymal cell proliferation or ploidy changes vs. mitoses in hepatocytes.

1   Clearly, the increases in liver weight that were reported as early as 5 days of exposure could not
2   have resulted from increased hepatocyte proliferation.
3
4   The H&E sections were reported to have been fixed in an aqueous solution that reduced
5   glycogen content. However, residual PAS positive material (assumed to be glycogen) was
6   reported to be present indicating that not all of the glycogen had been dissolved.   The authors
7   report changes in pathology between 5 and 30 days in control animals that included straightening
8   of hepatocyte cording, decreased mitoses, less clarity and more fine granularity of pericentral
9   hepatocellular cytoplasm, increased numbers of larger nuclei that were not labeled, and reported
10 differences between animals in the amount of glycogen present (i.e., 2 or 3 animals out of the 5
11 had less glycogen than other members of the group with less glycogen in the central and
12 midzonal areas).   These changes are consistent with increased polyploidization expected for
13 maturing mice (see Sections 1.1 and 1.2 above). After 5 days of treatment, 0.5 g/l exposed
14 animals were reported to have livers with fewer mitoses and tritiated thymidine hepatocyte
15 labeling but by 10 days an increase in nuclear size. Labeling was reported to be predominantly in
16 small nuclei. Animals given 0.5 g/l DCA for 15, 20, and 25 days were reported to have "focal
17 cells in the middle zone with less detectable or no cell membranes and loss of the coarse
18 granularity of the cytoplasm" with some cells not having nuclei or cells having a loss of nuclear
19 membrane and apparent karyolysis." "Cells without nuclei because the plane of the section did
20 not pass through the nuclei had the same type of nuclei. Cells without nuclei not related to plane
21 of section had a condensed cytoplasm."   Livers from 20-day and later sacrifice groups treated
22 with 0.5 g/l DCA were reported to have normal architecture. After 25 days of treatment
23 apoptotic bodies were reported to be observed with fewer nuclei around the central veins nuclei
24 that were larger in central and midzonal areas. In animals treated with 5 g/l DCA the authors
25 report similar features as for 0.5 g/l but in a zonal pattern. Inflammatory cells were reported to
26 not be observed and after 5 and 10 days a marked decrease in labeled nuclei. After 5 days of 5
27 g/l DCA, nuclear depletion in the central and mid-zonal areas was reported. In methyl green
28 pyronin-stained slides a marked loss of cellular membranes was reported at 5 days with a loss of
29 nuclei and formation of "lakes of liver cell debris."   After 15 days of treatment there was a
30 reported increase in labeling in comparison to animals sacrificed after 5 or 10 days. The cells
31 nearest to the triads were reported to have clearing of their cytoplasms and an increase in PAS
32 positivity. Hepatocytes of both 0.5 and 5 g/l DCA treatment groups were reported to have
33 "enlarged, presumably polyploidy nuclei." Some of the nuclei were reported to be "labeled,
34 usually in hepatocytes in the mid-zonal area."
35
36   The morphometric analyses of liver sections were reported to reveal statistically
37 significant changes in cellularity, nuclear size (as measured by either nuclear area or mean
38 diameter of the nuclear area equivalent circle), and multinucleated cells during 30 days exposure
39 to DCA. The authors reported that the concentration of total DNA in the liver, reported as total
40 μg nuclear DNA/ g liver, ranged between 278.17 ± 16.88 and 707.00 ± 25.03 in the control
41 groups (i.e., 2-5 fold range). No 0.5 g/l DCA treatment groups differed from their control group
42 in terms of liver DNA concentration.   However, for 10 though 30 days of exposure hepatic DNA
43 concentrations were reported to be decreased in the 5 g/l treatment groups (at 5 days there

1 appeared to be ~ 30% increase over control). The number of cells per field was reported to range
2 between 24.28 ± 1.94 and 43.81 ± 1.93 in control livers (i.e., 1.8-fold range). From 5 to 15 days
3 the number of cells/field decreased with 0.5 g/l DCA treatment although only at day 15 was the
4 change statistically significant. From 20 to 30 days of treatment only the 30 day treatment
5 showed a slight decrease in cells/field and that change was statistically significant. After 5 days
6 of treatment, the number of cells/field was 1.6-fold of control, by 15 days reduced by ~20%, and
7 for 20 to 30 days continued to be reduced by as much as 40%. Although the authors reported
8 that the changes in cellularity and DNA concentration to be closely correlated, the patterns in the
9 number of cells/field varied in their consistency with those of DNA concentration (i.e., for days
10 5, 20 and 25 there direction of change with dose was similar between the two parameters but for
11 days 10, 15 and 30 were not). If changes in liver weight were due to hepatocellular hypertrophy,
12 the increased liver size would be matched by a decrease in liver DNA concentration and by the
13 number of cells/field. The large increases in liver/body weight induced by 5 g/l DCA were
14 matched by decreases in liver DNA concentration except for the 5 day exposure group. In
15 general, the small increases in liver/body weight consistently induced by 0.5 g/l treatment from
16 day 5 through 30 were not correlated with DNA concentrations or cells/field. The small number
17 of animal examined for these parameters (i.e., 4-5) and the highly variable control values limit
18 the power to accurately detect changes. The apparent dehydration in the animals treated at 5 g/l
19 DCA was cited by the authors for the transient increase in cellularity and DNA concentration in
20 the 5-day exposure group. However, drinking water consumption was reported to be similarly
21 reduced at all treatment periods for 5 g/l DCA-treated animals so that all groups would
22 experience the same degree of dehydration.
23
24   The percentage of mononucleated cells was reported as % of mononucleated hepatocytes
25 with results given as means but with no reports of variation within groups. The mean control
26 values were reported to range between 60% and 75% for phase I and between 58% and 71% for
27 phase II of the experiment (n= 4-5 animals per group). The % of mononucleated hepatocytes
28 was reported to be similar between control and DCA treatment groups at 5- and 10-day exposure.
29 At 15 days both DCA treatments were reported to give a similar increase in mononucleated
30 hepatocytes (~ 80 % vs. 60% in control) with only the 5 g/l DCA group statistically significant.
31 The increase in mononucleated cells reported for DCA treatment is similar in size to the
32 variation between control values. For phase II of the study, DCA treatment was reported to
33 increase the number of mononucleated cells in at all concentrations and exposure time periods in
34 comparison to control values. However, only the increases for the 5 g/l treatments at days 20
35 and 25, and the 0.5 g/l treatment at day 30 were reported to be statistically significant. Again,
36 small numbers of animals limit the ability to accurately determine a change. However, the
37 consistent reporting of an increasing number of mononucleated cells between 15 and 30 days
38 could be associated with clearance of mature hepatocytes as suggested by the report of DCA-
39 induced loss of cell nuclei.
40
41   Mean nuclear area was reported to range between 45 and 54 μ^2 in phase I and to range
42 between 41 and 48 μ^2 in phase II of the experiment with no variation in measurements given by
43 the authors. The only statistically significant differences reported between control and treated

1   groups in phase I was a decrease from 54 to ~ 42 μ^2 in the 0.5 g/l DCA 10 day treatment group
2   and a small increase from 50 to ~ 52 μ^2 15 day treatment group. Clearly the changes reported by
3   the authors as statistically significant did not show a dose-related pattern and were within the
4   range of variation reported between control groups. For phase II of the experiment both DCA
5   treatment concentrations were reported to induce a statistically significant increase the nuclear
6   area that was dose-related with the exception of day 30 in which the nuclear area was similar
7   between the 0.5 and 5 g/l treatment groups. The largest increase in nuclear area was reported at
8   20 days for the 5 g/l treatment group (~ 72 vs. 41 μ^2 for control). The patterns of increases in
9   nuclear area were correlated with those of increased percentage of mononucleated cells in phase
10   II of the study (20 – 30 days of treatment) as well as the small changes seen in phase I of the
11   experiment. An increase in nuclear cell area is consistent with increase polyploidization without
12   mitosis as cells are induced towards polyploidization. A decrease in the numbers of binucleate
13   cells in favor of mononucleate cells is consistent with clearance of mature binucleate hepatocyte
14   as well induction of further polyploidization of diploid or tetraploid binucleate cell to tetraploid
15   or octoploid mononucleate cells. The authors suggested that the "large hyperchromatic
16   mononucleated hepatocytes are tetraploid" and suggest that such increases in tetraploid cells
17   have also been observed with non-genotoxic carcinogens and with DEHP. In terms of increased
18   cellular granularity observed by the authors with DCA treatment, this result is also consistent
19   with a more differentiated phenotype (Sigal et al., 1999). Thus, these results for DCA are
20   consistent with a DCA induced change in polyploidization of the cells without cell proliferation.
21   The pattern of consistent increase in % liver/body weight induced by 0.5 g/l DCA treatment from
22   days 5 though 30 was not consistent with the increased numbers of mononucleate cells and
23   increase nuclear area reported from day 20 onward. The large differences in liver weight
24   induction between the 0.5 g/l treatment group and the 5 g/l treatment groups at all times studied
25   also did not correlate with changes in nuclear size and % of mononucleate cells. Thus, increased
26   liver weight was not a function of cellular proliferation, but probably included both aspects of
27   hypertrophy associated with polyploidization and increased glycogen deposition induced by
28   DCA. The similar changes reported after short-term exposure for both the 0.5 and 5 g/l exposure
29   concentration were suggested by the authors to indicate that the carcinogenic mechanism at both
30   concentrations would be similar. Furthermore, they suggest that although there is evidence of
31   cytotoxicity (e.g., loss of cell membranes and apparent apoptosis), the present study does not
32   support that the mechanism of DCA-induced hepatocellular carcinogenesis is one of regenerative
33   hyperplasia following massive cell death nor peroxisome proliferation as the 0.5 g/l exposure
34   concentration has been shown to increase hepatocellular lesions after 100 weeks of treatment
35   without concurrent peroxisome proliferation or cytotoxicity (DeAngelo et al., 1999).
36
37   *2.3.1.5. DeAngelo et al. 1989*
38
39   Various strains of rats and mice were exposed to TCA (12 and 31 mM) or DCA (16 and
40   39 mM) for 14 days with Sprague-Dawley rats and B6C3F1 mice exposed to an additional
41   concentration of 6 mM TCA and 8 mM DCA. Although noting that in a previous study that high
42   concentrations of chloracids, the authors did not measure drinking water consumption in this
43   study. This study exposed several strains of male rats and mice to TCA at two concentrations in

1   drinking water (12 mM and 31mM neutralized TCA) for 14 days. The conversion of mmols/l or
2   mM TCA is 5 g/l TCA, 2 g/l TCA and 1 g/l for 31 mM, 12 mM, and 6 mM TCA, respectively.
3   The conversion of mmols/l of mM DCA is 5 g/l DCA, 2 g/l DCA, and 1 g/l DCA for 39 mM, 16
4   mM and 8 mM DCA, respectively.   The strains of mice tested were Swiss-Webster, B6C3F1,
5   C57BL/6, and C3H and for rats were Sprague Dawley, Osborne Mendel, and F344. For the
6   F344 rat and B6C3F1 mice data from two separate experiments were reported for each. The
7   number of animals in each group was reported to be 6 for most experiments with the exception
8   of the Sprague-Dawley rats (n=3 at the highest dose of TCA and n=4 or 5 for the control and the
9   lower TCA dose), one study in B6C3F1 mice (n= 4 or 5 for all groups), and one study in F344
10 rats (n=4 for all groups). The body weight of the controls was reported to range from 269 to 341
11 g in the differing strains of rats (1.27-fold) and 21 to 28 g in the differing strains of mice (1.33-
12 fold) (age not reported). For % liver/body weight ratios the range was 4.4% to 5.6% in control
13 rats (1.27-fold) and 5.1 to 6.8% in control mice (1.33-fold).
14
15   As discussed in other studies, the determination of PCO activity appears to be highly
16 variable. This enzyme activity is often used as a proxy for peroxisome proliferation. For PCO
17 activity the range of activity in controls was much greater than for either body weight or %
18 liver/body weight. For rats there was a 2.8-fold difference in PCO control activity and in mice
19 there was a 4.6-fold difference in PCO activity. Between the two studies performed in the same
20 strain of rat (F344) there was a 2.83-fold difference in PCO activity between controls, and for the
21 two studies in the same strain of mouse (B6C3F1) there was a 3.14-fold difference in PCO
22 activity between controls. Not only were there differences between strains and experiments in
23 the same strain, but also differences in control values between species with a wider range of
24 values in the mice. The lowest level of PCO activity in control rats, expressed as nanomoles
25 NAD reduced/min/mg/protein, was 3.34 and for control mice was 1.40. The highest level
26 reported in control in rats was 9.46 and for control mice was 6.40.
27
28   These groups of rats and mice were exposed to 2 g/l NaCl, 2 g/l or 5 g/l TCA in drinking
29 water for 14 days and their PCO activity assayed. These doses of TCA did not affect body
30 weight except for the Sprague-Dawley rats which lost ~ 16% of their body weight. This was also
31 the same group in which only 3 rats survived treatment.   The Osborne-Mendel and F344 strains
32 did no exhibit loss of body weight or mortality due to TCA exposure. There was a large
33 variation in response to TCA exposure between the differing strains of rats and mice with a much
34 larger difference between the strains of mice. For the 3 rat strains tested there was a range
35 between 0% change and 2.38-fold of control for PCO activity at the 5 g/l TCA exposure. For the
36 2 g/l TCA exposure, there was a range of 0% change to 1.54-fold of control for PCO activity.
37 The Osborne-Mendel rats had 1.54-fold of control value for PCO activity at 2 g/l TCA and 2.38-
38 fold of control value for PCO activity reported at 5 g/l, exhibiting the most consistent increase in
39 PCO with increased dose of TCA. Two experiments were reported for F344 rats with one
40 reporting a 1.63-fold of control and the other a 1.79-fold of control value for 5 g/l TCA. Only
41 one of the F334 experiments also exposed rats to 2 g/l TCA and reported no change from control
42 values.
43

1   For the 4 strains of mice tested there was a range of 7.44- to 22.13-fold of control values
2   reported at the 5 g/l TCA exposures and 3.76- to 25.92-fold of control values at the 2 g/l TCA
3   exposures for PCO activity. For the C57BL/6 strain of mice there was little difference between
4   the 5 g/l and 2 g/l TCA exposures and a generally 3-fold higher induction of PCO activity by
5   TCA at the 5 g/l TCA exposure level than for the other mouse strains. Although there was a 2.5-
6   fold difference between the 5 g/l and 2 g/l TCA exposure dose, the difference in magnitude of
7   PCO activity between these doses ranged from 0.85-fold to 2.23-fold for all strains of mice. For
8   the B6C3F1 mice there was a difference between reported increases of PCO activity in the text
9   (i.e., reported as 9.59-fold of control) for one of the experiments and that presented graphically
10   in Figure 2 (i.e., 8.70-fold of control). Nevertheless in the two studies of B6C3 F1 mice, 5 g/l
11   TCA was reported to induce 7.78-fold of control and 8.70-fold of control for PCO activity, and 2
12   g/l TCA was reported to induce 5.56-fold of control and 4.70-fold of control for PCO activity.
13   For the two F344 rat studies in which ~ 200 mg/kg or 5 g/l TCA was administered for 10 or 14
14   days, there was 1.63-fold of control and 1.79-fold of control values reported for PCO activity.
15   Thus, for experiments in which the same strain and dose of TCA were administered, there was
16   not as large a difference in PCO response than between strains and species.
17
18   Whether increases in % liver/body weight ratios were similar in magnitude to increased
19   PCO activity can be assessed by examination of the differences in magnitude of increase over
20   control for the 5 g/l and 2 g/l TCA treatments in the varying rat strains and mouse strains. The
21   relationship in exposure concentration was a 2.5:1 ratio for the 5 g/l and 2 g/l doses. For rats
22   treatment of 5 g/l TCA to Sprague-Dawley rats resulted in a significant decrease in body weight
23   and therefore affected the magnitude of increase in % liver/body weight ratio for this group.
24   However, for the rest of the rat and mouse data, this dose was not reported to affect body weight
25   so that there is more confidence in the dose-response relationship. For the Sprague-Dawley rat
26   there was no change in the % liver/body weight ratio at 2 g/l but a 10% decrease at 5 g/l TCA
27   exposure with no change in PCO activity for either. However, for the Osborne-Mendel rats,
28   there was no change in % liver/body weight ratios for either exposure concentration of TCA, but
29   PCO activity was reported to be 1.54-fold of control at 2 g/l and 2.38-fold of control at 5 g/l
30   TCA. Thus there was a ratio of 2.5-fold increase in PCO activity between the 5 g/l and 2 g/l
31   treatment groups. For the F344 rats there was a 2-fold difference in liver weight increases (i.e.,
32   12% vs. 6% increase over control) between the two exposure concentrations but 1.6-fold of
33   control value for PCO activity at the 5 g/l TCA exposure concentration and no increase in PCO
34   activity at the 2 g/l level. Thus for the three strains of rats, there did not appear to be a consistent
35   correlation between liver weight induction by TCA and PCO activity.
36
37   For differing strains of mice, similar concentrations of TCA were reported to vary in the
38   induction of liver weight increases. The range of liver weight induction was 1.26-fold to 1.66-
39   fold of control values between the 4 strains of mice at 5 g/l TCA and 1.16-fold to 1.63-fold at 2
40   g/l TCA. In general, for mice the magnitudes of the difference in the increase in dose between
41   the 5 g/l and 2 g/ TCA exposure concentration (2.5-fold) was generally higher than the increase
42   % liver/body weight ratios at these doses. The differences in liver weight induction between the
43   2g/l and 5g/l doses were ~ 40% for the Swiss-Webster, C3H, and for one of the B6C3F1 mouse

1   experiments. For the C57BL/6 mouse there was no difference in liver weight induction between
2   the 2 g/l and 5g/l TCA exposure groups. For the other B6C3F1 mouse experiments there was a
3   2.5-fold greater induction of liver weight increase for the 5g/l TCA group than for the 2 g/l
4   exposure group (1.39-fold of control vs. 1.16-fold of control for % liver/body weight,
5   respectively).   For PCO activity the Swiss-Webster, C3H, and one of the B6C3F1 mouse
6   experiments were reported to have ~ 2-fold difference in the increase in PCO activity between
7   the two doses. For the other B6C3F1 mouse experiment there was only about a 50% increase
8   and for the C57BL/6 mouse data there was 15% less PCO activity induction reported at the 5 g/l
9   TCA dose that at the 2 g/l dose. None of the difference in increases in liver weight or PCO
10   activity in mice from the 2 g/l or 5g/l TCA exposures were of the same magnitude as the
11   difference in TCA exposure concentration (i.e., 2.5-fold) except for liver weight from the one
12   experiment in B6C3F1 mice. This is also the data used fore comparisons with the Sprague-
13   Dawely rat discussed below.
14
15   In regard to strain differences for TCA response in mice, there did not appear to be
16   correlations of the magnitude of 5 g/l TCA-induced changes in % liver/body weight ratio or PCO
17   activity, with the body weights reported for control mice for each strain. The control weights
18   between the 4 strains of mice varied from 21 to 28 grams. The strain with the greatest response
19   (C57Bl/6) for TCA-induced changes in % liver/body weight ratio (i.e., 1.66-fold of control) and
20   PCO activity (22.13-fold of control) had a mean body weight reported to be 26 grams for
21   controls. At this dose, the range of % liver/body weight for the other strains was reported to be
22   1.26- to 1.39-fold of control and the range of PCO activity reported to be of 7.48- to 8.71-fold of
23   control.
24
25   Of note is that in the literature, this study has been cited as providing evidence of
26   differences between rats and mice for peroxisomal response to TCA and DCA. Generally the
27   PCO data from the Sprague Dawley rats and B6C3F1 mice at the highest dose of TCA and DCA
28   have been cited. However, the Sprague-Dawley strain was reported to have greater mortality
29   from TCA at this exposure than the other strains tested (i.e., only 3 rats survived and provided
30   PCO levels) and a lower PCO response (no change in PCO activity over control) that the other
31   two strains tested in this study (i.e., Osborne-Mendel rats was reported to have had 2.38-fold of
32   control and the F344-had a 1.63-to1.79-fold of control for PCO activity after exposure to 5 g/l
33   TCA with no mortality).   The B6C3F1 mouse was reported to have a 7.78- or 8.71-fold of
34   control for PCO activity from 5 g/l TCA exposure. Certainly the male mouse is more responsive
35   to TCA induction of PCO activity. However, as discussed above there are large variations in
36   control levels of PCO activity and in the magnitude and dose-response of TCA-induction of PCO
37   activity between rat and mouse strains and between species. If is not correct to state that the rat is
38   refractory to TCA-induction of peroxisome activity.
39
40   Unfortunately, the authors chose the Sprague-Dawley rat (i.e., the most unresponsive
41   strain for PCO activity and most sensitive to toxicity) for studies for comparative studies
42   between DCA and TCA effects. The authors also tested for carnitine acetyl CoA transferase
43   (CAT) activity as a marker of peroxisomal enzyme response and took morphometric analysis of

peroxisome # and cytoplasmic volume for one liver section for each of two B6C3F1 mice of Sprague-Dawley rats from the 5 g/l TCA and 5 g/l DCA treatment groups. Only 6 electron micrograph fields were analyzed from each section (12 fields total) were analyzed without identification as to what area of the liver lobules they were being taken from. Hence there is a question as to whether the areas which are known to be peroxisome rich were assayed of not. Also as noted above, previous studies have indicate that such high concentration of DCA and TCA inhibit drinking water consumption and therefore raising issues not only about toxicity but also the dose which rats and mice received. The number of peroxisomes per 100 μm^3 and cytoplasmic volume of peroxisomes was reported to be 6.60 and 1.94%, respectively, for control rats, and 6.89 and 0.61% for control mice, respectively. For 5 g/l TCA and 5 g/l DCA the numbers of peroxisomes were reported to be increased to 7.14 and 16.75, respectively in treated Sprague Dawley rats. Thus there was 2.5-fold and 1.08-fold of control reported in peroxisome # for 5 g/l DCA and TCA, respectively. The cytoplasmic volume of peroxisomes was reported to be 2.80 % and 0.89% for 5 g/l DCA and 5 g/l TCA, respectively (i.e., a 1.44-fold of control and ~ 60% reduction for 5 g/l DCA and 5 g/l TCA, respectively). Thus, 5 g/l TCA was reported to slightly increase the number of peroxisomes and but decrease the % of the cytoplasmic volume occupied by peroxisome by half. For DCA the reported pattern was for both to increase. PCO activity was reported to increase by a similar magnitude as peroxisome # but not volume in the 5 g/l TCA treated Sprague-Dawley rats. However, although peroxisomal volume was reported to be cut nearly in half and for peroxisome number to be similar, 5 g/l TCA treatment was not reported to change PCO activity in the Sprague-Dawley rat.

For comparisons between DCA and TCA B6C3 F1 mice were examined at 1.0, 2.0, and 5.0 g/l concentrations. DCA was reported to induce a higher % liver/body weight ratio that did TCA at every concentration (i.e., 1.55-fold, 1.27-fold, 1.21-fold of control for DCA and 1.39-fold, 1.16-fold, and 1.08-fold of control for TCA at 1.0, 2.0, and 5.0 g/l concentrations, respectively). As noted above, for other strains of mice tested and a second experiment with B6C3F1 mice, there was 40% or less difference in % liver/body weight ratio between the 2.0 g/l and 5.0 g/l exposures to TCA but for this experiment there was a 2.5-fold difference. Thus, at 5 g/l there was ~ 40% greater induction of liver weight for DCA than TCA. In the B6C3F1 mice, 5 g/l TCA was reported to increase peroxisome number to 30.75 and cytoplasmic volume to 4.92% (i.e., 4.4-fold and 8.1-fold of control, respectively). For 5 g/l DCA treatment, the peroxisome number was reported to be 30.77 and 3.75% (i.e., 4.5-fold and 6.1-fold of control, respectively). While there was no difference in peroxisome number and ~ 40% difference in cytoplasmic volume at the 5.0 g/l exposures of DCA and TCA, there was a greater difference in the magnitude of PCO activity increase. The 5 g/l TCA exposure was reported to induce 4.3-fold of control for PCO activity while 5 g/l DCA induced as 9.6 –fold of control PCO activity (although a Figure in the report shows 8.7-fold of control) which is a ~ 2.5-fold difference between DCA and TCA at this exposure concentration.. Thus for one of the B6C3F1 mouse studies, 5 g/l DCA and TCA treatments were reported to give a similar increase peroxisome #, TCA to induce a 40% greater increase in peroxisomal cytoplasmic volume than DCA and a 2.5-fold greater increase in PCO activity, but DCA to induce ~ 40% greater liver weight induction than TCA.

1   Not only were PCO activity, peroxisome number and cytoplasmic volume occupied by
2   peroxisomes analyzed but also CAT activity as a measure of peroxisome proliferation. For TCA
3   and DCA the results were opposite those reported for PCO activity. In Sprague-Dawley rats
4   control levels of CAT were reported to be 1.81 nmoles of carnitine transferred/min/mg/protein.
5   Exposure to 5 g/l TCA was reported to increase CAT activity by 3.21-fold of control while 5 g/l
6   DCA was reported to induce CAT activity to 10.33-fold of control levels in Sprague-Dawley
7   rats. However, while PCO activity was reported to be the same as controls, and peroxisomal
8   volume decreased, 5 g/l TCA increased CAT activity 3.21-fold of control in these rats. The level
9   of CAT induced by 5 g/l DCA was over 10-fold of control in the rat while peroxisome # was
10   only 2.5-fold of control and cytoplasmic volume 1.4-fold of control. Thus, the fold increases for
11   these three measures were not the same for DCA treatment and for TCA in rats. Nevertheless for
12   CAT, DCA was a stronger inducer in rats than was TCA. In B6C3 F1 mice 5 g/l TCA and 5 g/l
13   DCA induced CAT activity to a similar extent (4.50-fold and 5.61-fold of control, respectively).
14   The magnitude of CAT induction was similar to that of peroxisome # for both 5 g/l DCA and 5
15   g/l TCA and lower than PCO activity in DCA-treated mice and cytoplasmic volume in TCA-
16   treated mice by about half. Thus, using CAT as the marker of peroxisome proliferation, the rat
17   was more responsive than the mouse to DCA and nearly as responsive to TCA as the mouse at
18   this high dose in these two specific strains. This, data illustrates the difficulty of using only one
19   measure for peroxisome proliferation and shows that the magnitude of increased PCO activity is
20   not necessarily predictive of the peroxisome # or cytoplasmic volume or CAT activity. The
21   difficulty of interpretation of the data from so few animals and sections for the electron
22   microscopy analysis, and the low number of animals for PCO activity and CAT activity (n= 3 to
23   6), the high dose studied (5 g/l), and the selection of a rat strain that appears to be more resistant
24   to this activity but more susceptible to toxicity than the others tested, should be taken into
25   account before conclusions can be made about differences between these chemicals for
26   peroxisome activity between species.
27
28   Of note is that PCO activity was also shown to be increased by corn oil alone in F344 rats
29   and to potentiate the induction of PCO activity of TCA. After 10 days of exposure to either
30   water, corn oil, 200 mg/kg/day TCA in corn oil or 200 mg/kg TCA in water via gavage dosing,
31   there was 1.40-fold PCO activity from corn oil treatment alone in comparison to water, a 1.79-
32   fold PCO activity from TCA in water treatment in comparison to water, and a 3.14-fold PCO
33   activity from TCA in corn oil treatment in comparison to water.
34
35   The authors provided data for 3 concentrations of DCA and TCA for Sprague-Dawley
36   and for one experiment in the B6C3F1 mouse for examination of changes in body and %
37   liver/body weight ratios (1, 2, or 5 g/l DCA or TCA) after 14 days of exposure. As noted above,
38   not only did the 5 g/l exposure concentration of DCA result in mortality in the Sprague-Dawley
39   strain of rat, but the 5 g/l and 2 g/l concentrations of DCA were reported to decrease body weight
40   (~ 20 and 25%, respectively). The 5 g/l dose of TCA was also reported to induce a statistically
41   significant decrease in body weight in the Sprague-Dawley rat. There were no differences in
42   final body weight in any of the mice exposed to TCA or DCA. As noted above no TCA or DCA
43   exposure group of Sprague-Dawley rats was reported to have a statistically significant increase

1   in % liver/body weight ratio over control. For the B6C3F1 male mice, the % liver/body weight
2   ratio was 1.22-fold, 1.27-fold and 1.55 fold of control after exposure to 1 g/l, 2 g/l and 5 g/l
3   DCA, respectively, and 1.08-fold, 1.16-fold, and 1.39-fold of control after exposure to 1 g/l, 2
4   g/l, and 5 g/l TCA, respectively. Thus, for DCA there was only a 20% increase in liver weight
5   corresponding to the 2-fold increase between the 1 and 2 g/l exposure levels of DCA. Between
6   the 2 g/l and 5 g/l exposure concentrations of DCA there was a 2-fold increase in liver weight
7   corresponding to a 2.5-fold increase in exposure concentration. For TCA, the magnitude of
8   increase in dose was reported to be proportional to the magnitude of increase in % liver/body
9   weight ratio in the B6C3 F1 male mouse. As stated above, the correspondence between
10 magnitude of dose and % liver weight for TCA exposure in this experiment differed from the
11 other experiment reported for this strain of mouse and also differed from the other 3 strains of
12 mice examined in this study where the magnitude in liver weight gain was much less than
13 exposure concentration.
14
2.3.2. Subchronic and Chronic Studies of DCA and TCA
16
17   Several experiments have been conducted with exposure to DCA and TCA, generally at
18 very high levels with a limited dose range, for less periods of time than standard carcinogenicity
19 bioassays, and with very limited information on any endpoints other than the liver tumor
20 induction. Caldwell and Keshava (2006) and Caldwell et al. (2008b) have examined these
21 studies for inferences of modes of action for TCE. Key studies are briefly described below for
22 comparative purposes of results reported in TCE studies.
23
24 *2.3.2.1. Snyder et al. 1995*
25
26   Studies of TCE have reported either no change or a slight increase in apoptosis only after
27 a relatively high exposure level (Dees and Travis, 1993; Channel et al., 1998). Inhibition of
28 apoptosis, which has been suggested to prevent removal of "initiated" cells from the liver and
29 lead to increased survival of precancerous cells, has been proposed as part of the MOA for
30 peroxisome proliferators (see Section 3.4). The focus of this study was to examine whether
31 DCA, which has been shown to inhibit DNA synthesis after an initial transient increase (see
32 Section 2.3.3 below), also alters the frequency of spontaneous apoptosis in mice. This study
33 exposed 28-day old male B6C3F1 male mice (n=5) to 0, 0.5 or 5.0 g/l buffered DCA in drinking
34 water for up to 30 days (phase I = 5-15 days exposure and phase II = 20-30 days treatment).
35 Portions of the left lobe of the liver were prepared for histological examination after H&E
36 staining. Hepatocyte number was determined by counting nuclei in 50 fields with
37 nonparenchymal cell nuclei excluded on the basis of nuclear size. Multinucleate cells were
38 counted as one cell. Apoptotic cells were visualized by *in situ* TDT nick end-labeling (TUNEL)
39 assay from 2-4 different liver sections from each control or treated animal. The average number
40 of apoptotic cells was then determined for each animal in each group. The authors reported that
41 in none of the tissues examined were necrotic foci observed, there was no any indication of
42 lymphocyte or neutrophil infiltration indicative of an inflammatory response, and suggested that
43 no necrotic cells contributed to the responses in their analysis.

Control animals were reported to exhibit apoptotic frequencies ranging from ~ 0.04 to 0.085% and that over the 30-day period the frequency rate declined. The authors suggested that this result is consistent with reports of the livers of these young animals undergoing rapid changes in cell death and proliferation. They note that animals receiving 0.5 g/l DCA also had a similar trend of decreasing apoptosis with age, supportive of the decrease being a physiological phenomenon. The 0.5 g/l exposure level of DCA was reported to decrease the percentage of apoptotic hepatocytes as the earliest time point studied and to remain statistically significantly decreased from controls from 5 to 30 days of exposure. The rate of apoptosis ranged from ~ 0.025 to 0.060% after 0.5 g/l DCA exposure during the 30-day period (i.e. and ~ 30-40 % reduction). Animals receiving the 5.0 g/l DCA dose exhibited a significant reduction at the earliest time point that was sustained at a similar level and statistically significant throughout the time-course of the experiment (% apoptosis ranged from 0.015 – 0.030%).   The results of this study not only provides a baseline of apoptosis in the mouse liver, which is very low, but also to show the importance of taking into account the effects of age on such determinations. The authors reported that the for rat liver the estimated frequency of spontaneous apoptosis to be ~ 0.1% and therefore greater than that of the mouse. The significance of the DCA-induced reduction in apoptosis, of a level that is already inherently low in the mouse, for the MOA for induction of cancer is difficult to discern.

2.3.2.2. Mather et al. 1990

This 90-day study in male Sprague-Dawley rats examined the body and organ weight changes, liver enzyme levels, and PCO activity in livers from rats treated with estimated concentrations of 3.9, 35.5, 345 mg/kg day DCA or 4.1, 36.5, or 355 mg/kg/day TCA from drinking water exposures (i.e., 0, 50, 500 and 5000 ppm or 0.05, 0.5, or 5.0 g/l DCA or TCA in the drinking water). All dose levels of DCA and TCA were reported to result in a dose-dependent decrease in fluid intake at 2 months of exposure. The rats were 9 (DCA) or 10 (TCA) weeks old at the beginning of the study (n= 10/group). Animals with body weights that varied more than 20% of mean weights were discarded from the study. The DCA and TCA solutions were neutralized. The mean values for initial weights of the animals in each test group varied less than 3%. DCA treatment induced a dose-related decrease in body weight that was statistically significant at the two highest levels (i.e., a 6%, 9.5%, and 17% decrease from control). TCA treatment also resulted in lower body weights that were not statistically significant (i.e., 2.1%, 4.4%, and 5.9%). DCA treatments were reported to result in a dose-related increase in absolute liver weights (1.01-fold, 1.13-fold, and 1.36-fold of control that were significantly different at the highest level) and % liver/body weight ratios (1.07-fold, 1.24-fold, and 1.69-fold of control that were significant at the two highest dose levels).   TCA treatments were reported to not result in changes in either absolute liver weights or % liver/body weight ratios with the exception of statistically significant increase in % liver/body weight ratios at the highest level of treatment (1.02-fold of control). Total serum protein levels were reported to be significantly depressed in all animals treated with DCA with animals in the two highest dose groups also exhibiting elevations of alkaline phosphatase. Alanine-amino transferase levels were reported to be elevated only in the highest treatment group. No consistent treatment-related

1   effect on serum chemistry was reported to be observed for the TCA-treated animals with data not
2   shown. In terms of PCO activity, there was only a mild increase at the highest dose of 15% for
3   TCA and a 2.5-fold level of control for DCA treatment that were statistically significant. The
4   difference in PCO activity between control groups for the DCA and TCA experiments was
5   reported to be 33%. No treatment affect was reported to be apparent for hepatic microsomal
6   enzymes, or measures of immunotoxicity for either DCA or TCA but data were not shown.
7   Focal areas of hepatocellular enlargement in both DCA- and TCA-treated rats were reported to
8   be present with intracellular swelling more severe with the highest dose of DCA treatment.
9   Livers from DCA treated rats were reported to stain positively for PAS, indicating significant
10 amounts of glycogen with TCA treated rats reported to display "less evidence of glycogen
11 accumulation." Of note is that, in this study of rats, DCA was reported to induce a greater level
12 of PCO activity than did TCA.

14 *2.3.2.3. Parrish et al. 1996*

16   Parrish et al. (1996) exposed male B6C3F1 mice (8 weeks old and 20-22 g upon
17 purchase) to TCA or DCA (0, 0.01, 0.5 and 2.0 g/l) for 3 or 10 weeks (n= 6). Livers were
18 excised and nuclei isolated for examination of 8-OHdG and homogenates examined for cyanide
19 insensitive Acyl-CoA oxidase (PCO) and laurate hydroxylase activity. The authors noted that
20 control values between experiments varied as much as a factor of 2-fold for PCO activity and
21 that data were presented as percent of concurrent controls. Initial body weights for treatment
22 groups were not presented and thus differences in mean values between the groups cannot be
23 ascertained.

25   Final body weights were reported to not be statistically significantly changed by DCA or
26 TCA treatments at 21 days or 71 days of treatment (all were within ~ 8% of controls). The mean
27 % liver/body ratios were reported to be 5.4%, 5.3%, 6.1%, and 7.2% for control, 0.1 g/l, 0.5 g/l ,
28 and 2.0 g/l TCA, respectively and 5.4%, 5.5%, 6.7%, and 7.9% for control, 0.1 g/l, 0.5 g/l and
29 2.0 g/l DCA, respectively after 21 days of exposure. This represents 0.98-fold, 1.13-fold, 1.33-
30 fold of control levels with these exposure levels of TCA and 1.02-fold, 1.24-fold, and 1.46-fold
31 of control levels with DCA after 21 days of exposure. For 71 days of exposure the mean %
32 liver/body ratios were reported to be 5.1%, 4.6%, 5.8%, and 6.9% for control, 0.1 g/l, 0.5 g/l ,
33 and 2.0 g/l TCA, respectively and 5.1%, 5.1%, 5.9%, and 8.5% for control, 0.1 g/l, 0.5 g/l and
34 2.0 g/l DCA, respectively. This represents 0.90-fold, 1.14-fold, 1.35-fold of control with TCA
35 exposure and 1.0-fold, 1.15-fold, and 1.67-fold of control with DCA exposure after 71 days of
36 exposure. The magnitude of difference between the 0.1 and 0.5 g/l TCA doses is 5 and 0.5 and
37 2.0 g/l doses is 4-fold. For the 21-day and 71-day exposures the magnitudes of the increases in
38 % liver/body weight over control values were greater for DCA than TCA exposure at same
39 concentration with the exception of 0.5 g/l doses at 71 days in which both TCA and DCA
40 induced similar increases. For TCA, the 0.01 g/l dose produces a similar 10% decrease in %
41 liver/body weight. Although there was a 4-fold increase in magnitude between the 0.5 and 2.0
42 g/l TCA exposure concentrations, the magnitude of increase for % liver/body weight increase
43 was 2.5-fold between them at both 21 and 71 days of exposure. For DCA, the 0.1 g/l dose was

1   reported to have a similar value as control for % liver/body weight ratio.   Although there was a
2   4-fold difference in dose between the 0.5 and 2.0 g/l DCA exposure concentrations, there was a
3   ~ 2-fold increase in % liver/body weight increase at 21 days and ~ 4.5-fold increase at 71 days.
4
5   As a percentage of control values, TCA was reported to induce a dose-related increase in
6   PCO activity at 21 days (~ 1.5-fold, 2.2-fold, and ~ 4.1-fold of control, for 0.1, 0.5, and 2.g/l
7   TCA exposures). Only the 2.0 g/l dose of DCA was reported to induce a statistically significant
8   increase at 21-days of exposure of PCO activity over control (~ 1.8-fold of control) with the 0.1
9   and 0.5 g/l exposure PCO activity to be slightly less than control values (~ 20% less). Thus,
10   although there was no increase in % liver/body weight at 0.1 g/l TCA, the PCO activity was
11   reported to be increased by ~50% after 21 days. A 13% increase in liver weight at 0.5 g/l TCA
12   was reported to be associated with 2.2-fold of control level of PCO activity and a 33% increase
13   in liver weight after 2.0 g/l TCA to be associated with 4.1-fold of control level of PCO activity.
14   Thus, increases in PCO activity were not necessarily correlated with concurrent TCA-induced
15   increases in liver weight and the magnitudes of increase in liver weight between 0.5g/l and 2.0
16   g/l TCA (2.5-fold) was greater than the corresponding increase in PCO activity (1.8-fold of
17   control). Although there was a 20-fold difference in TCA dose, the magnitude of increase in
18   PCO activity between 0.1 and 2.0 g/l TCA was ~ 2.7-fold. As stated above, the 4-fold difference
19   in TCA dose at the two highest levels resulted in a 2.5 fold increase in liver weight. For DCA,
20   the increases in liver weight at 0.1 and 0.5 g/l DCA exposures were not associated with increased
21   PCO activity after 21 days of exposure. The 2.0 g/l DCA exposure concentration was reported to
22   induce 1.8-fold of control PCO activity. After 71 days of treatment, TCA induced a dose-related
23   increase in PCO activity that was ~ twice the magnitude as that reported at 21 days (i.e. ~ 9-fold
24   greater at 2.0 g/l level). After 71 days, for DCA the 0.1 and 0.5 g/l doses produced a statistically
25   significant increase in PCO activity (~ 1.5-fold and 2.5-fold of control, respectively). The
26   administration of 1.25 g/l clofibric acid in drinking water was used as a positive control and
27   reported to induce ~6-7-fold of control PCO activity at 21 and 71 days of exposure.
28
29   Laurate hydroxylase activity was reported to be elevated significantly only by TCA at 21
30   days (2.0g/l TCA dose only) and to increased to approximately the same extent (~ 1.4 to 1.6-fold
31   of control values) at all doses tested. For 0.1 g/l DCA the laurate hydroxylase activity was
32   reported to be similar to that of 0.1 g/l TCA (~ 1.4-fold of control) but to be ~ 1.2-fold of control
33   at both the 0.5 and 2.0 g/l DCA exposures. At 71 days, both the 0.5 and 2.0 g/l TCA exposures
34   induced a statistically significant increase in laurate hydroxylase activity (i.e., 1.6-fold and 2.5-
35   fold of control, respectively) with no change after DCA exposure. The actual data rather than %
36   of control values were reported for laurate hydroxylase activity. The control values for laurate
37   hydroxylase activity varied 1.7-fold between 21 and 71 days experiments. The results for 8-
38   OHdG levels are discussed in Section 3.4.2.3 below. Of note is that the increases in PCO
39   activity noted for DCA and TCA were not associated with 8-OHdG levels (which were
40   unchanged, see Section 3.4.2.3 below) and also not with changes laurate hydrolase activity or %
41   liver/body weight ratio increases observed after either DCA or TCA exposure. A strength of this
42   study is that is examined exposure concentrations that were lower than those examined in many
43   other short-term studies of DCA and TCA.

1 *2.3.2.4 Bull et al. 1990*

2

3   The focus of this study was the determination of "dose-response relationships in the
4 tumorigenic response to these chemicals [sic DCA and TCA] in B6C3F1 mice, determine the
5 nature of the non-tumor pathology that results from the administration of these compounds in
6 drinking water, and test the reversibility of the response." Male and female B6C3F1 mice (age
7 37 days) were treated from 15 to 52 weeks with neutralized TCA and TCA. A highly variable
8 number and generally low number of animals were reported to be examined in the study with
9 n=5 for all time periods except for 52 weeks where in males the n= 35 for controls, n=11 for 1
10 g/L DCA, n = 24 for 2 g/L DCA, n= 11 for 1g/L TCA, and n= 24 for 2 g/L TCA exposed mice.
11 Female mice were only examined after 52 weeks of exposure and the number of animals
12 examined was n= 10 for control, 2g/L DCA, and 2 g/L TCA exposed mice. "Lesions to be
13 examined histologically for pathological examination were selected by a random process" with
14 lesions reported to be selected from 31 of 65 animals with lesions at necropsy. 73 of 165 lesions
15 identified in 41 animals were reported to be examined histologically.   All hyperplastic nodules,
16 adenomas and carcinomas were lumped together and characterized as hepatoproliferative lesions.
17 Accordingly there were only exposure concentrations available for dose-response analyses in
18 males and only "multiplicity of hepatoproliferative lesions" were reported from random samples.
19 Thus, this data cannot be compared to other studies and is unsuitable for dose-response with
20 inadequate analysis performed on random samples for pathological examination. The authors
21 state that some of the lesions taken at necropsy and assumed to be proliferative were actually
22 histologically normal, necrotic, or an abscess as well. It is also limited by a relatively small
23 number of animals examined in regard to adequate statistical power to determine quantitative
24 differences. Similar concerns were raised by Caldwell et al (2007b) with a subsequent study
25 (e.g., Bull, 2002).   For example, the authors report that 5/11 animals had "lesions" at 1g/L TCA
26 at 52 weeks and 19/24 animals had lesions at 2 g/L TCA at 52 weeks. However, while 7 lesions
27 were examined in 5 mice bearing lesions at 1 g/L TCA, only 16 of 30 lesions from 11 of the 19
28 animals bearing lesions examined in the 2 g/L TCA group. Therefore, almost half of the mice
29 with lesions were not examined histologically in that group along with only half of the "lesions."
30

31   The authors reported the effects of DCA and TCA exposure on liver weight and %
32 liver/body changes (m ± SEM) and these results gave a pattern of hepatomegaly generally
33 consistent with short-term exposure studies. The authors report "no treatment produced
34 significant changes in the body weight or kidney weight of the animals (data not shown)" In
35 male mice (n = 5) at 37 weeks of exposure, liver weights were reported to be 1.6 ± 0.1, 2.5 ± 0.1,
36 and 1.9 ± 0.1 g for control, 2 g/l DCA, and 2 g/l TCA exposed mice, respectively. The %
37 liver/body weights were reported to be 4.1 ± 0.3, 7.3 ± 0.2, and 5.1. ± 0.1 % for control, 2 g/l
38 DCA, and 2 g/l TCA exposed mice, respectively. In male mice at 52 weeks of exposure, liver
39 weights were reported to be 1.7 ± 0.1, 2.5 ± 0.1, 5.1 ± 0.1 , 2.2 ± 0.1, and 2.7 ± 0.1 g for control
40 (n = 35), 1 g/l DCA (n = 11), 2 g/l DCA (n= 24), 1 g/l TCA (n = 11), and 2 g/l TCA (n = 24)
41 exposed mice, respectively. In male mice at 52 weeks of exposure, % liver/body weights were
42 reported to be 4.6 ± 0.1, 6.5 ± 0.2, 10.5 ± 0.4, 6.0 ± 0.3, and 7.5 ± 0.5 % for control, 1 g/l DCA,
43 2 g/l DCA, 1 g/l TCA, and 2 g/l TCA exposed mice, respectively. For female mice (n = 10) at

1 52 weeks of exposure, liver weights were reported to be 1.3 ± 0.1, 2.6 ± 0.1, and 1.7 ± 0.1 g for
2 control, 2 g/l DCA, and 2 g/l TCA exposed mice, respectively. The % liver/body weights were
3 reported to be 4.8 ± 0.3, 9.0 ± 0.2, and $6.0. \pm 0.3$ % for control, 2 g/l DCA, and 2 g/l TCA
4 exposed mice, respectively. Although the number of animals examined varied 3-fold between
5 treatment groups in male mice, the authors reported that all DCA and TCA treatments were
6 statistically increased over control values for liver weight and % body/liver weight in both
7 genders of mice. In terms of % liver/body weight ratio, female mice appeared to be as
8 responsive as males at the exposure concentration tested. Thus, hepatomegaly reported at these
9 exposure levels after short-term exposures appeared to be further increased by chronic exposure
10 with equivalent levels of DCA inducing greater hepatomegaly than TCA.
11
12   Interestingly, after 37 weeks of treatment and then a cessation of exposure for 15 weeks
13 liver weights were assessed in control male mice, 2 g/l DCA treated mice, and 2 g/l TCA treated
14 mice (n = 11 for each group but results for controls were pooled and therefore n = 35). Liver
15 weights were reported to be 1.7 ± 0.1, 2.2 ± 0.1, and 1.9 ± 0.1 g for control, 2 g/l DCA, and 2 g/l
16 TCA exposed mice, respectively. The % liver/body weights were reported to be 4.6 ± 0.1, $5.7 \pm$
17 0.3, and $5.4. \pm 0.2$ % for control, 2 g/l DCA, and 2 g/l TCA exposed mice, respectively. After 15
18 weeks of cessation of exposure, liver weight and % liver/body weight were reported to still be
19 statistically significantly elevated after DCA or TCA treatment. The authors partially attribute
20 the remaining increases in liver weight to the continued presence of hyperplastic nodules in the
21 liver. The authors state that because of the low incidence of lesions in the control group and the
22 two groups that had treatments suspended, all the lesions from these groups were included for
23 histological sectioning. However, the authors present a table indicating that, of the 23 lesions
24 detected in 7 mice exposed to DCA for 37 weeks, 19 were examined histologically. Therefore,
25 groups that were exposed for 52 weeks had a different procedure for tissue examination as those
26 at 37 weeks. In terms of liver tumor induction, the authors stated that "statistical analysis of
27 tumor incidence employed a general linear model ANOVA with contrasts for linearity and
28 deviations from linearity to determine if results from groups in which treatments were
29 discontinued after 37 weeks were lower than would have been predicted by the total dose
30 consumed." The multiplicity of tumors observed in male mice exposed to DCA or TCA at 37
31 weeks and then sacrificed at 52 weeks were reported by the authors to have a response in animals
32 that received DCA very close to that which would be predicted from the total dose consumed by
33 these animals. The response to TCA was reported by the authors to deviate significantly (P=
34 0.022) from the linear model predicted by the total dose consumed. Multiplicity of lesions per
35 mouse and not incidence was used as the measure. Most importantly the data used to predict the
36 dose response for "lesions" used a different methodology at 52 weeks than those at 37 weeks.
37 Not only were not all animal's lesions examined but foci, adenomas, and carcinomas were
38 combined into one measure. Therefore foci, of which a certain percentage have been commonly
39 shown to spontaneously regress with time, were included in the calculation of total "lesions".
40 Pereira and Phelps (1996) note that in initiated mice treated with DCA, the yield of altered
41 hepatocytes decreases as the tumor yields increase between 31 and 51 weeks of exposure
42 suggesting progression of foci to adenomas. Initiated and non-initiated control mice also had
43 fewer foci/mouse with time. Because of differences in methodology and the lack of discernment

1   between foci, adenomas, and carcinomas for many of the mice exposed for 52 weeks, it is
2   difficult to compare differences in composition of the "lesions" after cessation of exposure. For
3   TCA treatment the number of animals examined for determination of which "lesions" were foci,
4   adenomas, and carcinomas was 11 out of the 19 mice with "lesions" at 52 weeks while all 4 mice
5   with lesions after 37 weeks of exposure and 15 weeks of cessation were examined. For DCA
6   treatment the number of animals examined was only 10 out of 23 mice with "lesions" at 52
7   weeks while all 7 mice with lesions after 37 weeks of exposure and 15 weeks of cessation were
8   examined. Most importantly, when lesions were examined microscopically then did not all turn
9   out to be preneoplastic or neoplastic. Two lesions appeared "to be histologically normal" and
10   one necrotic. Not only were a smaller number of animals examined for the cessation exposure
11   than continuous exposure but only the 2 g/l exposure levels of DCA and TCA were studied for
12   cessation. The number of animals bearing "lesions" at 37 and then 15 week cessation weeks was
13   7/11 (64%) while the number of animals bearing lesions at 52 weeks was 23/24 (96%) after 2 g/l
14   DCA exposure. For TCA the number of animals bearing lesions at 37 weeks and then 15 weeks
15   cessation was 4/11 (35%) while the number of animals bearing lesions at 52 weeks was 19/24
16   (80%). While suggesting that cessation of exposure diminished the number of "lesions,"
17   conclusions regarding the identity and progression of those lesion with continuous vs. non-
18   continuous DCA and TCA treatment are tenuous.
19
20   Macroscopically, the "livers of many mice receiving DCA in their drinking water
21   displayed light colored streaks on the surface" at every sacrifice period and "corresponded with
22   multi-focal areas of necrosis with frequent infiltration of lymphocytes." At the light microscopic
23   level, the lesions were described to also be present in the interior of the liver as well. For TCA-
24   treated mice, "similar necrotic lesions were also observed… but at a much lower frequency,
25   making it difficult to determine if they were treatment-related." Control animals were reported
26   not to show degenerative changes. "Marked cytomegaly" was reported for mice treated with
27   either 1 or 2 g/l DCA "throughout the liver"   In regard to cell size the authors did not give any
28   description in the methods section of the paper as to how sections were selected for
29   morphometric analysis or what areas of the liver acinus were examined but reported after 52
30   weeks of treatment the long axis of hepatocytes measured (mean ± S.E.) 24.9 ± 0.3, 38.5 ± 1.0,
31   and 29.3 ± 1.4 μm in control, DCA- and TCA- treated mice, respectively.
32
33   Mice treated with TCA (2 g/l) for 52 weeks were reported to have livers with
34   "considerable dose-related accumulations of lipofuscin." However, no quantitative analyses
35   were presented. A series of figures representative of treatment showed photographs (1000X) of
36   lipofuscin fluorescence indicating greater fluorescence in TCA treated liver than control or DCA
37   treated liver.
38
39   A series of photographs of H&E sections in the report (Figures 2 a, b and c) are shown as
40   representative histology of control mice, mice treated with 2 g/l DCA and 2 g/l TCA. The area
41   of the liver from which the photographs were taken did not include either portal tract or central
42   veins and the authors did not give the zone of the livers from which they were taken. The figure
43   representing TCA treatment shows only a mild increase in cell volume in comparison to controls,

1   while for DCA treatment the hepatocyte diameter was greatly enlarged, pale stained so that
2   cytoplasmic contents appear absent, nuclei often pushed to the cell perimeter, and the sinusoids
3   appearing to be obscured by the swollen hepatocytes. The apparent reduction of sinusoidal
4   volume by the enlarged hepatocytes raises the possibility of decreased blood flow through the
5   liver which may have been linked to focal areas of necrosis reported for this high exposure level.
6   In a second set of figures Glycogen accumulation was shown with PAS staining at the same level
7   of power (400X) for the same animals. In control animals PAS positive material was not
8   uniformly distributed between or within hepatocytes but send to show a zonal pattern of
9   moderate intensity. PAS positive staining (which the authors reported to be glycogen) appeared
10   to be slightly less than controls but with a similar pattern in the photograph representing TCA
11   exposure. However, for DCA the photograph showed a uniform and heavy stain within each
12   hepatocyte and across all hepatocytes.   The authors stated in the results section of the paper that
13   "the livers of TCA-treated animals displayed less evidence of glycogen accumulation and it was
14   more prominent in periportal than centrilobular portions of the liver acinus." In their abstract
15   they state "TCA produced small increases in cell size and a much more modest accumulation of
16   glycogen." Thus, the statement in the text, which is suggestive that TCA induced an increase in
17   glycogen over controls that was not as much as that induced by DCA, and the statement in the
18   abstract which concludes TCA exposure increased glycogen is not consistent with the
19   photographs. In the photograph shown for TCA there is less not more PAS positive staining
20   associated with TCA treatment in comparison to controls. In Sanchez and Bull (1990) the
21   authors report that "TCA exposure induced a much less intense level of PAS staining that was
22   confined to periportal areas" but do not compare PAS staining to controls but only to DCA
23   treatment. In the discussion section of the paper the authors state "Except for a small increase in
24   liver weight and cell size, the effects produced by DCA were not observed with TCA."   Thus,
25   there seems to be a discrepancy with regard to what the effects of TCA are in relation to control
26   animals from this report.that has caused confusion in the literature. Kato-Weinstein et al (2001)
27   reported that in male mice exposed to DCA and TCA the DCA increased glycogen and TCA
28   decreased glycogen content of the liver using chemical measurement of glycogen in liver
29   homogenates and using ethanol-fixed sections stained with PAS, a procedure designed to
30   minimize glycogen loss).

32   *2.3.2.5.   Nelson et al. 1990*

34   Nelson et al (1990) reported that they used the same exposure paradigm as Herren-
35   Freund et al. (1987), with little description of methods used in treatment of the animals. Male
36   B6C3F1 mice were reported to be exposed to DCA (1 or 2 g/l) or TCA (1 or 2 g/l) for 52 weeks.
37   The number of animals examined for non-tumor tissue was 12 for controls. The number of
38   animals varied from 2 to 8 for examination of non-tumor tissue, hyperplastic nodules, and
39   carcinoma tissues for c-Myc expression.   There was no description for how hyperplastic nodules
40   were defined and whether they included adenomas and foci. For the 52-week experiments, the
41   results were pooled for lesions that had been obtained by exposure to the higher or lower
42   concentrations of DCA or TCA (i.e., the TCA results are for lesions induced by either 1.0 g/l or
43   2.0 g/l TCA). A second group of mice were reported to be given either DCA or TCA for 37

1   weeks and then normal drinking water for the remaining time till 52 weeks with no
2   concentrations given for the exposures to these animals. Therefore it is impossible to discern
3   what dose was used for tumors analyzed for c-Myc expression in the 37-week treatment groups
4   and if the same dose was used for 37 and 52 week results. Autoradiography was described for 3
5   different sections per animal in 5 different randomly chosen high power fields per section. The
6   number of hyperplastic nodules or the number of carcinomas per animal induced by these
7   treatments was not reported nor the criteria for selection of lesions for c-myc expression.
8   Apparently a second experiment was performed to determine the expression of c-H-ras.
9   Whereas in the first experiment there were no hyperplastic nodules, in the second 1 control
10   animal was reported to have a hyperplastic nodule. The number of control animals reported to
11   be examined for non-tumor tissue in the second group was 12. The numbers of animals in the
12   second group was reported to vary from 1 to 7 for examination of non-tumor tissue, hyperplastic
13   nodules, and carcinoma tissues for c-H-ras expression. The number of animals per group for the
14   investigation of H-ras did not match the numbers reported for that of c-Myc. The number of
15   animals treated to obtain the "lesion" results was not presented (i.e., how many animals were
16   tested to get a specific number of animals with tumors that were then examined). The number of
17   lesions assessed per animal was not reported.
18
19   At 52 weeks of exposure, hyperplastic nodules (n= 8 animals) and carcinomas (n= 6
20   animals) were reported to have ~ 2-fold expression of c-Myc relative to non-tumor tissue (n= 6
21   animals) after DCA treatment. After 37 weeks of DCA treatment and cessation of exposure,
22   there was a ~ 30% increase in c-Myc in hyperplastic nodules (n=4 animals) that was not
23   statistically significant. There were no carcinomas reported at this time. After 52 weeks of TCA
24   exposure, there was ~ 2-fold of non-tumor tissue reported for c-Myc in hyperplastic nodules (n=
25   6 animals) and ~ 3-fold reported for carcinomas (n=6 animals). After 37 weeks of TCA
26   exposure there was ~ 2-fold c-Myc in hyperplastic nodules (n= 2 animals) that was not
27   statistically significant and ~ 2.6 fold increase in carcinomas (n=3 animals) that was reported to
28   be statistically significant over non-tumor tissue. There was no difference in c-Myc expression
29   between untreated animals and non-tumor tissue in the treated animals.
30
31   The authors reported that c-Myc expression in TCA-induced carcinomas was "almost 6
32   times that in control tissue (corrected by subtracting nonspecific binding)," and concluded that c-
33   Myc in TCA-induced carcinomas was significantly greater than in hyperplastic nodules or
34   carcinomas and hyperplastic nodules induced by DCA. However the c-myc expression reported
35   as the number of grains per cells was ~ 2.6-fold in TCA-induced carcinomas and ~ 2-fold in
36   DCA-induced carcinomas than control or non-tumor tissue at 52 weeks. The hyperplastic
37   nodules from DCA- and TCA-treatments at 52 weeks gave identical ratios of ~ 2-fold   In 3
38   animals per treatment, c-Myc expression was reported to be similar in "selected areas of high
39   expression" for either DCA or TCA treatments of 52 weeks.
40
41   There did not appear to be a difference in c-H-ras expression between control and non-
42   tumor tissue from DCA- or TCA- treated mice. The levels of c-H-ras transcripts were reported
43   to be "slightly elevated" in hyperplastic nodules induced by DCA (~ 67%) or TCA (~ 43%) but

1 these elevations were not statistically significant in comparison to controls. However,
2 carcinomas "derived from either DCA- or TCA-treated animals were reported to have
3 significantly increased c-H-ras levels relative to controls." The fold increase of non-tumor tissue
4 at 52 weeks for DCA-induced carcinomas was ~2.5-fold and for TCA induced carcinomas ~2.0
5 fold. Again the authors state that "if corrected for non-specific hybridization, carcinomas
6 expressed approximately 4 times as much c-H-ras than observed in surrounding tissues" Given
7 that control and non-tumor tissue results were given as the controls for the expression increases
8 observed in "lesions", it is unclear what this the usefulness of this "correction" is. The authors
9 reported that "focal areas of increased expression of c-H-ras were not observed within
10 carcinomas."
11
12   The limitations of this experiment include uncertainty as to what doses were used and
13 how many animals were exposed to produce animals with derive tumors. In addition results of
14 differing doses were pooled and the term hyperplastic nodule, undefined.   The authors state that
15 c-Myc expression in itself is not sufficient for transformation and that its over expression
16 commonly occurs in malignancy.   They also state that "Unfortunately, the limited amount of
17 tissue available prevented a more serious pursuit of this question in the present study." In regard
18 to the effects of cessation of exposure, the authors do not present data on how many animals
19 were tested with the cessation protocol, what doses were used, and how many lesions comprised
20 their results and thus comparisons between these results and those from 52 weeks of continuous
21 exposure are hard to make. Quantitatively, the small number of animals, whose lesions were
22 tested, was 2-4 for the cessation groups. Bull et al. (1990) is given as the source of data for the
23 cessation experiment (see Section 2.3.2.1 above).
24
25 *2.3.2.6. DeAngelo et al. 1999*
26
27   The focus of this study was to "determine a dose response for the hepatocarcinogenicity
28 of DCA in male mice over a lifetime exposure and to examined several modes of action that
29 might underlie the carcinogenic process." As DeAngelo et al pointed out, many studies of DCA
30 had been conducted at high concentrations and for less than lifetime studies, and therefore of
31 suspect relevence to environmental concentrations. This study is one of the few that examined
32 DCA at a range of exposure concentrations to determine a dose-response in mice.   The authors
33 concluded that DCA-induced carcinogenesis was not dependent on peroxisome proliferation or
34 chemically sustained proliferation. The number of hepatocellular carcinomas/animals was
35 reported to be significantly increased over controls at all DCA treatments including 0.05 g/l and
36 a NOEL not observed. Peroxisome proliferation was reported to be significantly increased at 3.5
37 g/L DCA only at 26 weeks and did not correlate with tumor response. No significant treatment
38 effects on labeling of hepatocytes (as a measure of proliferation) outside proliferative lesions
39 were also reported and thus that DCA-induced liver cancer was not dependent on peroxisome
40 proliferation or chemically sustained cell proliferation.
41
42   Male B6C3F1 mice were 28-30 days of age at the start of study and weighed 18-21 grams
43 (or ~ 14% range). They were exposed to 0, 0.05, 0.5, 1.0, 2.0, and 3.5 g/l DCA via drinking

water as a neutralized solution. The time-weighted mean daily water consumption calculated over the 100-week treatment period was reported to be 147, 153, 158, 151, 147, and 124 (84% of controls) ml/kg/day for 0, 0.05, 0.5, 1, 2, and 3.5 g/l DCA, respectively. The number of animals reported as used for interim sacrifices were 35, 30, 30, 30 and 30 for controls, 0.5 g/l, 1.0 g/l, 2.0 g/l, and 3.5 g/l DCA treated groups respectively (i.e., 10 mice per treatment group at interim sacrifices of 26, 52 and 78 weeks). The number of animals at final sacrifice were reported to be 50, 33, 24, 32, 14 and 8 for controls, 0.05g/l, 0.5 g/l, 1.0 g/l, 2.0 g/l, and 3.5 g/l DCA treated groups respectively The number of animals with unscheduled deaths before final sacrifice were reported to be 3, 2, 1, 9, 11 and 8 for controls, 0.05g/l, 0.5 g/l, 1.0 g/l, 2.0 g/l, and 3.5 g/l DCA treated groups respectively. The Authors reported that early mortality tended to occur from liver cancer. The number of animals examined for pathology were reported to be 85, 33, 55, 65, 51 and 41 for controls, 0.05g/l, 0.5 g/l, 1.0 g/l, 2.0 g/l, and 3.5 g/l DCA treated groups respectively The experiment was conducted in two parts with control, 0.5 g/l , 1.0 g/l, 2.0 g/l and 3.5 g/l groups treated and then 1 months later a second group consisting of 30 control group mice and 35 mice in a 0.05 g/l DCA exposure group studied. The authors reported not difference in prevalence and multiplicity of hepatocellular neoplasms in the two groups so that data were summed and reported together. The # of animals reported as examined for tumors were n=10 animals, with controls reported to be 35 animals split among 3 interim sacrifice times – exact number per sacrifice time is unknown. The number of animals reported "with pathology" and assumed to be included in the tumor analyses from Table 1, and the sum of the number of animals "scheduled for sacrifice that survived till 100 weeks" and "interim sacrifices" do not equal each other. For the 1 g/l DCA exposure group, 30 animals were sacrificed at interim periods, 32 animals were sacrificed at 100 weeks, 9 animals were reported to have unscheduled deaths, but of those 71 animals only 65 animals were reported to have pathology for the group. Therefore some portion of animals with unscheduled deaths must have been included in the tumor analyses. The exact number of animals that may have died prematurely but included in analyses of pathology for the 100 week group is unknown. In Figure 3 of the study, the authors reported prevalence and multiplicity of hepatocellular carcinomas following 79 to 100 weeks of DCA exposure in their drinking water. The number of animals in each dose group used in the tumor analysis for 100 weeks was not given by the authors. Given that the authors included animals that survived past the 78 interim sacrifice period but died unscheduled deaths in their 100 week results, the number must have been greater than those reported as present at final sacrifice. A comparison of the data for the 100-week data presented in Table 3a and Figure 3 shows that the data reported for 100 weeks is actually for animals that survived from 79 to 100 weeks. The authors report a dose-response that is statistically significant from 0.5 to 3.5 g/l DCA for hepatocellular carcinoma incidence and a dose-response in hepatocellular carcinoma multiplicity that is significantly increased over controls from 0.05 to 0.5 g/l DCA that survived 79 to 100 weeks of exposure (i.e., 0, 8-, 84-, 168-, 315- and 429 mg/kg/day dose groups with prevalences of 26%, 33%, 48%, 71%, 95%, and 100%, respectively, and multiplicities of 0.28, 0.58, 0.68, 1.29, 2.47, and 2.90, respectively). Hepatocellular adenoma incidence or multiplicity was not reported for the 0.05 g/l DCA exposure group.

1   In Table 3 of the report, the time course of hepatocellular carcinomas and adenoma
2   development are given and summarized below.
3
4   Prevalence   Multiplicity
5   (lesions/animal m± SEM)
6   Carcinomas   Adenomas
7
8   52 weeks control = 0% carcinomas, 0% adenoma   0   0
9   0.5 g/L DCA = 0/10 carcinoma, 1/10 adenomas   0 0.10 ± 0.09
10   1.0 g/L DCA = 0/10 carcinomas, 1/10 adenomas   0 0.10 ± 0.09
11   2.0 g/L DCA = 2/10 carcinomas, 0/10 adenomas 0.20 ± 0.13 0
12   3.5 g/L DCA = 5/10 carcinomas, 5/10 adenomas 0.70 ± 0.25 0.80 ± 0.31
13
14   78 weeks control = 10% carcinomas, 10% adenomas 0.10 ± 0.10 0.10 ± 0.09
15   0.5 g/L DCA = 0/10carcinoma, 1/10 adenomas   0 0.10 ± 0.09
16   1.0 g/L DCA = 2/10 carcinomas, 2/10 adenomas 0.20 ± 0.13 0.20 ± 0.13
17   2.0 g/L DCA = 5/10 carcinomas, 5/10 adenomas 1.0 ± 0.47 1.00 ± 0.42
18   3.5 g/L DCA = 7/10 carcinomas, 5/10 adenomas 1.20 ± 0.37 1.00 ± 0.42
19
20   100 weeks control = 26% carcinoma, 10% adenoma 0.28 ± 0.07 0.12 ± 0.05
21   0.5 g/L DCA = 48% carcinoma, 20% adenomas 0.68 ± 0.17 0.32 ± 0.14
22   1.0 g/L DCA = 71% carcinomas, 51.4% adenomas 1.29 ± 0.17 0.80 ± 0.17
23   2.0 g/L DCA = 95% carcinomas, 42.9% adenomas 2.47 ± 0.29 0.57 ± 0.16
24   3.5 g/L DCA = 100% carcinomas, 45% adenomas 2.90 ± 0.40 0.64 ± 0.23
25
26   The authors reported hepatocellular carcinomas and number of lesions/animal in mice
27   that survived 79-100 wk of exposure (they combined exposure groups to be animals after the
28   week 78 sacrifice time that did and did not make it to 100 weeks). This is the same data reported
29   above for the 100 week exposure with the inclusion of the 0.05 g/l DCA data. The difference
30   between number of animals at interim and final sacrifices and those "with pathology" and used
31   in the tumor analysis but most likely coming from unscheduled deaths is reported below as
32   "extra" and varied across treatment groups.
33

Dose = Prevalence of HC	#HC/animal	N = at 100weeks	extra added in
Control = 26%	0.28	50	0
0.05 g/L = 33%	0.58	33	0
0.5 g/L = 48%	0.68	24	1
1 g/L= 71%	1.29	32	3
2 g/L= 95%	2.47	14	7
3.5 g/L = 100%	2.90	8	3

41
42   These data show a dose-related increase in tumor formation and decrease in time-to-
43   tumor associated with DCA exposure at the lowest levels examined. These findings are limited

1   by the small number of animals examined at 100 weeks but especially those examined at
2   "interim sacrifice" periods (n= 10). The data illustrate the importance of examining multiple
3   exposure levels at lower concentrations at longer durations of exposure and with an adequate
4   number of animals to determine the nature of a carcinogenic response.
5
6   Preneoplastic and non-neoplastic hepatic changes were reported to have been described
7   previously and summarized as large preneoplastic foci observed at 52 weeks with multiplicities
8   of 0.1, 0.1, 0.2 and 0.16 for 0.5 g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA exposure respectively. At 100
9   weeks all values were reported to be significant (0.03, 0.06, 0.14, 0.27 for 0.5 g/l, 1 g/l, 2 g/l, and
10   3.5 g/l DCA exposure respectively. Control values were not reported by the authors. The
11   authors reported that the prevalence and severity of hepatocellular cytomegaly and of
12   cytoplasmic vacuolization with glycogen deposition to be dose-related and considered significant
13   in all dose groups examined when compared to control liver. However, no quantitative data was
14   shown. The authors reported a severity index of 0 = none, 1 = $\leq 25\%$, 2 = $50\text{-}75\%$ and 4 = 75%
15   of liver section for hepatocellular necrosis and report at 26 weeks scores (n= 10 animals) of 0.10
16   $\pm$ 0.10, 0.20 $\pm$ 0.13, 1.20 $\pm$ 0.38, 1.20 $\pm$ 0.39 and 1.10 $\pm$ 0.28 for control, 0.5g/l, 1 g/l, 2 g/l and
17   3.5 g/l DCA treatment groups, respectively. Thus there appeared to be a treatment but not dose-
18   related increase in hepatocellular necrosis that is does not involve most of the liver from 1 g/l to
19   3.5 g/l DCA at this time point. At 52 weeks of exposure the score for hepatocellular necrosis
20   was reported to be 0, 0, 0.20 $\pm$ 0.13, 0.40 $\pm$ 0.22 and 1.10 $\pm$ 0.43 for control, 0.5g/l, 1 g/l, 2 g/l
21   and 3.5 g/l DCA treatment groups, respectively.   At 78 weeks of exposure the score for
22   hepatocellular necrosis was reported to be 0, 0, 0, 0.30 $\pm$ 0.21 and 0.20 $\pm$ 0.13 for control, 0.5g/l,
23   1 g/l, 2 g/l and 3.5 g/l DCA treatment groups, respectively. Finally, the final sacrifice time when
24   more animals were examined the extent of hepatocellular necrosis was reported to be 0.20 $\pm$
25   0.16, 0.20 $\pm$ 0.08, 0.42 $\pm$ 0.15, 0.38 $\pm$ 0.20 and 1.38 $\pm$ 0.42 for control, 0.5g/l, 1 g/l, 2 g/l and 3.5
26   g/l DCA treatment groups, respectively. Thus, there was not reported increase in hepatocellular
27   necrosis at any exposure period for 0.5g/l DCA treatment and the mild hepatocellular necrosis
28   seen at the three highest exposure concentrations at 26 weeks had diminished with further
29   treatment except for the highest dose at up to100 weeks of treatment. Clearly the pattern of
30   hepatocellular necrosis did not correlate with the dose-related increases in hepatocellular
31   carcinomas reported by the authors and was not increased over control at the 0.5 g/l DCA level
32   where there was a DCA-related tumor increase.
33
34   The authors cite previously published data and state that cyanamid –insensitive
35   palmitoyl coenzyme A (CoA) oxidase activity (a marker of peroxisome proliferation) data for the
36   26 week time point plotted against 100 wk hepatocellular carcinoma prevalence of animals
37   bearing tumors was significantly enhanced at concentrations of DCA that failed to induce
38   "hepatic PCO" activity. The authors report that neither 0.05 nor 0.5 g/l DCA had any marked
39   effect on PCO activity and that it was "only significantly increased after 26 weeks of exposure to
40   3.5 g/l DCA and returned to control level at 52 weeks (data not shown)." In regards to
41   hepatocyte labeling index after treatment for 5 days with tritiated thymidine, the authors report
42   that animals examined in the dose-response segment of the experiment at 26 and 52 weeks were
43   examined but no details of the analysis were reported.   The authors comment on the results from

1　this study and a previous one that included earlier time points of study and stated that there were
2　"no significant alterations in the labeling indexes for hepatocytes outside of proliferative lesions
3　at any of the DCA concentrations when compared to the control values with the exception of
4　0.05 g/l DCA at 4 weeks (4.8 ± 0.6 vs. 2.7 ± 0.4 control value; data not shown)."
5
6　　　　The effects of DCA on body weight, absolute liver weight and % liver/body weight were
7　given in Table 2 of the paper for 26, 52, 78 and 100 weeks exposure. For 52 and 78 week studies
8　10 animals per treatment group were examined. Liver weights were not determined for the
9　lowest exposure concentration (0.05 g/l DCA) except for the 100 week exposure period. At 26
10　weeks of exposure there was not a statistically significant change in body weight among the
11　exposure groups (i.e., 35.4 ± 0.7, 37.0 ± 0.8, 36.8 ± 0.8, 37.9 ± 0.6, and 34.6 ± 0.8 g for control,
12　0.5 g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA, respectively). Absolute liver weight was reported to have a
13　dose-related significant increase in comparison to controls at all exposure concentrations
14　examined with liver weight reaching a plateau at the 2 g/l concentration (i.e., 1.86 ± 0.07, $2.27 \pm$
15　0.10, 2.74 ± 0.08, 3.53 ± 0.07, and 3.55 ± 0.1 g for control, 0.5 g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA,
16　respectively). The % liver/body weight ratio increases due to DCA exposure were reported to
17　have a similar pattern of increase (i.e., 5.25 ± 0.11, 6.12 ± 0.16, 7.44 ± 0.12, 9.29 ± 0.08, and
18　10.24 ± 0.12 % for control, 0.5 g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA, respectively). This represented
19　a 1.17-fold, 1.41-fold, 1.77-fold, and 1.95-fold of control % liver/body weight at these exposures
20　at 26 weeks.
21
22　　　　At 52 weeks of exposure there was not a statistically significant change in body weight
23　among the exposure groups except for the 3.5 g/l exposed group in which there was a significant
24　decrease in body weight (i.e., 39.9 ± 0.8, 41.7 ± 0.8, 41.7 ± 0.9, 40.8 ± 1.0, and 35.0 ± 1.1 g for
25　control, 0.5 g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA, respectively). Absolute liver weight was reported
26　to have a dose-related significant increase in comparison to controls at all exposure
27　concentrations examined with liver weight reaching a plateau at the 2 g/l concentration (i.e., 1.87
28　± 0.13, 2.39 ± 0.04, 2.92 ± 0.12, 3.47 ± 0.13, and 3.25 ± 0.24 g for control, 0.5 g/l, 1 g/l, 2 g/l,
29　and 3.5 g/l DCA, respectively). The % liver/body weight ratio increases due to DCA exposure
30　were reported to have a similar pattern of increase (i.e., 4.68 ± 0.30, 5.76 ± 0.12, 7.00 ± 0.15,
31　8.50 ± 0.26, and 9.28 ± 0.64 % for control, 0.5 g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA, respectively).
32　For liver weight and % liver/body weight there was much larger variability between animals
33　within the treatment groups compared to controls and other treatment groups. There were no
34　differences reported for patterns of change in body weight, absolute liver weight, and %
35　liver/body weight between animals examined at 26 weeks and those examined at 52 weeks. At
36　78 weeks of exposure there was not a statistically significant change in body weight among the
37　exposure groups except for the 3.5 g/l exposed group in which there was a significant decrease in
38　body weight (i.e., 46.7 ± 1.2, 43.8 ± 1.5, 43.4 ± 0.9, 42.3 ± 0.8, and 40.2 ± 2.2 g for control, 0.5
39　g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA, respectively). Absolute liver weight was reported to have a
40　dose-related increase in comparison to controls at all exposure concentrations examined but none
41　were reported to be statistically significant (i.e., 2.55 ± 0.14, 2.16 ± 0.09, 2.54 ± 0.36, $3.31 \pm$
42　0.63, and 3.93 ± 0.59 g for control, 0.5 g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA, respectively). The %
43　liver/body weight ratio increases due to DCA exposure were reported to have a similar pattern of

1   increase over control values but only the 3.5 g/l exposure level was reported to be statistically
2   significant (i.e., 5.50 ± 0.35, 4.93 ± 0.09, 5.93 ± 0.97, 7.90 ± 1.55, and 10.14 ± 1.73 % for
3   control, 0.5 g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA, respectively). Finally, for the animals reported to
4   be sacrificed between 90 and 100 weeks there was not a statistically significant change in body
5   weight among the exposure groups except for the 2.0 and 3.5 g/l exposed groups in which there
6   was a significant decrease in body weight (i.e., 43.9 ± 0.8, 43.3 ± 0.9, 42.1 ± 0.9, 43.6 ± 0.7, 36.1
7   ± 1.2 g, and 36.0 ± 1.3 g for control, 0.05 g/l, 0.5 g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA, respectively).
8   Absolute liver weight did not show a dose-response pattern at the two lowest exposure levels but
9   was elevated with the 3 highest doses with the two highest being statistically significant (i.e.,
10 2.59 ± 0.26, 2.74 ± 0.20, 2.51 ± 0.24, 3.29 ± 0.21, 4.75 ± 0.59 and 5.52 ± 0.68 g for control, 0.05
11 g/l, 0.5 g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA, respectively).   The % liver/body weight ratio increases
12 due to DCA exposure were reported to have a similar pattern of increase over control values but
13 only the 2.0 and 3.5 g/l exposure levels were reported to be statistically significant (i.e., 6.03 $\pm$
14 0.73, 6.52 ± 0.55, 6.07 ± 0.66, 7.65 ± 0.55, 13.30 ± 1.62 and 15.70 ± 2.16 % for control, 0.05 g/l,
15 0.5 g/l, 1 g/l, 2 g/l, and 3.5 g/l DCA, respectively).

16
17   It must be recognized that liver weight increases, especially in older mice, will reflect
18 increased weight due to tumor burden and thus DCA-induced hepatomegaly will be somewhat
19 obscured at the longer treatment durations. However, by 100 weeks of exposure there did not
20 appear to be an increase in liver weight at the 0.05 g/l and 0.5 g/l exposures while there was an
21 increase in tumor burden reported. Examination of the 0.5 g/l exposure group from 26 to 100
22 weeks shows that slight hepatomegaly, reported as either absolute liver weight increase over
23 control or change in % liver/body ratio, was present by 26 weeks (i.e., 22% increase in liver
24 weight and 17% increase in %liver/body weight), decreased with time, and while similar at 52
25 weeks, was not significantly different from control values at 78 or 100 weeks durations of
26 exposure. However, tumor burden was increased at this low concentration of DCA.

27
28   The authors present a figure comparing the number of hepatocellular carcinomas per
29 animal at 100 weeks compared with the % liver/body weight at 26 weeks and show a linear
30 correlation ($r^2 = 0.9977$). Peroxisome proliferation and DNA synthesis, as measured by tritiated
31 thymidine, were reported to not correlate with tumor induction profiles and were also not
32 correlated with early liver weight changes induced by DCA exposure. Most importantly, in a
33 paradigm that examined tumor formation after up to 100 weeks of exposure, DCA-induced
34 tumor formation was reported to occur at concentrations that did not also cause cytotoxicity and
35 at levels 20 to 40 times lower than those used in "less than lifetime" studies reporting concurrent
36 cytotoxicity.

37
38 *2.3.2.7. Carter et al. 2003*

39
40   The focus of this study was to present histopathological analyses that included
41 classification, quantification and statistical analyses of hepatic lesions in male B6C3F1 mice
42 receiving DCA at doses as low as 0.05g/l for 100 weeks and at 0.5, 1.0, 2.0, and 3.5 g/l for
43 between 26 and 100 weeks. This analysis used tissues from the DeAngelo et al.. (1999) (two

1   blocks from each lobe and all lesions found at autopsy). This study used the following
2   diagnostic criteria for hepatocellular changes. Altered hepatic Foci (AHF) were defined as
3   histologically identifiable clones that were groups of cells smaller than a liver lobule that did not
4   compress the adjacent liver. Large Foci of Cellular Alteration (LFCA) were defined as lesions
5   larger than the liver lobule that did not compress the adjacent architecture (previously referred to
6   as hyperplastic nodules by Bull et al., 1990) but had different staining. These are not non-
7   neoplastic proliferative lesions termed "hepatocellular hyperplasia" that occur secondary to
8   hepatic degeneration or necrosis. Adenomas (ADs) showed growth by expansion resulting in
9   displacement of portal triad and had alterations in both liver architecture and staining
10   characteristics. Carcinomas (CAs) were composed of cells with a high nuclear-to-cytoplasmic
11   ration and with nuclear pleomorphism and atypia that showed evidence of invasion into the
12   adjacent tissue. They frequently showed a trabecular pattern characteristic of mouse
13   hepatocellular CAs.
14

15   The report grouped lesions as eosinophilic, basophilic and/or clear cell, and dysplastic.
16   "Eosinophilic lesions included lesions that were eosinophilic but could also have clear cell,
17   spindle cell or hyaline cells. Basophilic lesions were grouped with clear cell and mixed cell (i.e.,
18   mixed basophilic, eosinophilic, hyaline, and/or clear cell) lesions." The authors reported that
19   "this grouping was necessary because many lesions had both a basophilic and clear cell
20   component and a few <10 % had an eosinophilic or hyaline component." "Lesions with foci of
21   cells displaying nuclear pleomorphism, hyperchromasia, prominent nucleoli, irregular nuclear
22   borders and/or altered nuclear to cytoplasmic ratios were considered dysplastic irrespective of
23   their tinctorial characteristics." Therefore, Carter et al. (2003) lumped mixed phenotype lesions
24   into the basophilic grouping so that comparisons with the results of Bull et al. (2002) or Pereira
25   (1996), which segregate mixed phenotype from those without mixed phenotype, cannot be done.
26

27   This report examined type and phenotype of preneoplastic and neoplastic lesions pooled
28   across all time points. Therefore, conclusions regarding what lesions were evolving into other
29   lesions have left out the factor of time. Bannasch (1996) reported that examining the evolution
30   of foci through time is critical for discerning neoplastic progression and described foci evolution
31   from eosinophilic or basophilic lesions to more basophilic lesions. Carter et al. (2003) suggest
32   that size and evolution into a more malignant state are associated with increasing basophilia, a
33   conclusion consistent with those of Bannasch (1996). The analysis presented by Carter et al
34   (2003) also suggested that there was more involvement of lesions in the portal triad which may
35   give an indication where the lesions arose. Consistent with the results of DeAngelo et al (1999),
36   Carter et al. (2003) reported that "DCA (0.05 – 3.5 g/l) increased the number of lesions per
37   animal relative to animals receiving distilled water and shortened the time to development of all
38   classes of hepatic lesions." They also concluded that "although this analysis could not
39   distinguish between spontaneously arising lesions and additional lesions of the same type
40   induced by DCA, only lesions of the kind that were found spontaneously in control liver were
41   found in increased numbers in animals receiving DCA." "Development of eosinophilic,
42   basophilic and/or clear cell and dysplastic AHF was significantly related to DCA dose at 100
43   weeks and overall adjusted for time." The authors concluded that the presence of isolated,

1　highly dysplastic hepatocytes in male B6C3F1 mice chronically exposed to DCA suggested
2　another direct neoplastic conversion pathway other than through eosinophilic or basophilic foci.
3
4　　　It appears that the lesions being characterized as carcinomas and adenomas in DeAngelo
5　et al (1999) were not the same as those by Carter et al. (2003) at 100 weeks even though they
6　were from the same tissues. Carter et al. identified all carcinomas as dysplastic despite tincture
7　of lesion and subdivided adenomas by tincture. If the differing adenoma multiplicities are
8　summed for Carter et al. they do not add up to the same total multiplicity of adenoma given by
9　DeAngelo et al. It is unclear how many animals were included in the differing groups in both
10　studies for pathology. The control and high-dose groups differ in respect to "animals with
11　pathology" between DeAngelo et al. and the "number of animals in groups" examined for lesions
12　in Carter et al. Neither report gave how many animals with unscheduled deaths were treated in
13　regards to how the pathology data was included in presentation of results. Given that DeAngelo
14　et al. represents animals at 100 weeks as also animals from 79 -100 weeks exposure, it is
15　probable that the animals that died after 79 weeks were included in the group of animals
16　sacrificed at 100 weeks. However, the number of animals affecting that result (which would be a
17　mix of exposure times) for either DeAngelo et al., or Carter et al., is unknown from published
18　reports. In general, it appears that Carter et al. (2003) reported more adenomas/animal for their
19　100 week animals than DeAngelo et al. (1999) did, while DeAngelo et al. reported more
20　carcinomas/animal. Carter et al. reported more adenomas/animal than controls while DeAngelo
21　et al. reported more carcinomas/animal than controls at 100 weeks of exposure.
22
23　　　In order to compare this data with others (e.g., Pereira, 1996) for estimates of multiplicity
24　by phenotype tincture it would be necessary to add foci and LFCA together as foci, and
25　adenomas and carcinomas together as tumors. It would also be necessary to lump mixed foci
26　together as "basophilic" from other data sets as was done for Carter et al. in describing
27　"basophilic lesions". If multiplicity of carcinomas and adenomas are summed from each study
28　to control for differences in identification between adenoma and carcinoma, there are still
29　differences in the two studies in multiplicity of combined lesions/animal with DeAngelo giving
30　consistently higher estimates. However, both studies show a dose response of tumor multiplicity
31　with DCA and a difference between control values and the 0.05 DCA exposure level. Error is
32　introduced by having to transform the data presented as a graph in Carter et al. (2003).   Also no
33　SEM is given for the Carter data.
34
35　Comparison of data from Carter et al. (2003) and DeAngelo et al. (1999):
36

Exposure level of DCA at 79-100 weeks (g/L)	Total adenoma multiplicity (Carter)	Total adenoma multiplicity (DeAngelo)	Total carcinoma multiplicity (Carter)	Total carcinoma multiplicity (De Angelo)	Sum of adenomas and carcinoma multiplicity (Carter)	Sum of adenomas and carcinoma multiplicity (DeAngelo)
0	0.22	0.12	0.05	0.28	0.27	0.40
0.05	0.48	-	<0.025	0.58	~0.50	-

0.5	0.44	0.32	0.20	0.68	0.64	1.0
1.0	0.52	0.80	0.30	1.29	0.82	2.09
2.0	0.60	0.57	1.55	2.47	2.15	3.27
3.5	1.48	0.64	1.30	2.90	2.78	3.54

1
2
3   In regard to other histopathological changes, the authors report that "necrosis was found
4   in 11.3% of animals in the study and the least prevalent toxic or adaptive response. No focal
5   necrosis was found at 0.5 g/L. The incidence of focal necrosis did not differ from controls at 52
6   or 78 weeks and only was greater than controls at the highest dose of 3.5 g/L at 100 weeks.
7   Overall necrosis was negatively related to the length of exposure and positively related to the
8   DCA dose. Necrosis was an early and transitory response. There was no difference in necrosis 0
9   and 0.05 g/L or 0.5 g/L. There was an increase in glycogen at 0.5 g/L at the perioportal area.
10 There was no increase in steatosis but a dose-related decrease in steatosis. Dysplastic LFCA
11 were not related to necrosis indicating that these lesions do not represent, regenerative or
12 reparative hyperplasia. Nuclear atypia and glycogen accumulation were associated with
13 dysplastic adenomas. Necrosis was not related to occurrence of dysplastic adenomas. Necrosis
14 was of borderline significance in relation to presence of hepatocellular carcinomas. Necrosis
15 was not associated with dysplastic LFCAs or Adenomas." They concluded that "the degree to
16 which hepatocellular necrosis underlies the carcinogenic response is not fully understood but
17 could be significant at higher DCA concentrations ($\geq$ 1g/L)."
18
19 *2.3.2.8. Stauber and Bull 1997*
20
21   This study was designed to examine the differences in phenotype between altered hepatic
22 foci and tumors induced by DCA and TCA. Male B6C3F1 mice (7 weeks old at the start of
23 treatment) were treated with 2.0 g/l neutralized DCA or TCA in drinking water for 38 or 50
24 weeks, respectively. They were then treated with additional exposures (n = 12) of 0, 0.02, 0.1,
25 0.5, 1.0, 2.0 g/L DCA or TCA for an additional 2 weeks. Three days prior to sacrifice in DCA-
26 treated mice or 5 days for TCA-treated mice, animals had miniosmotic pumps implanted and
27 administered BrdU. Immunohistochemical staining of hepatocytes from randomly selected
28 fields (minimum of 2000 nuclei counter per animal) from 5 animals per group were reported for
29 14- and 28-day treatments. It was unclear how many animals were examined for 280- and 350-
30 day treatments from the reports. The percentage of labeled cells in control livers was reported to
31 vary between 0.1 and 0.4 % (i.e., 4-fold). There was a reported ~ 3.5-fold of control level for
32 TCA labeling at 14 day time period and a ~ 5.5 fold for DCA. At 28 days there was ~ 2.5 fold of
33 control for TCA but a ~ 2.3-fold decrease of control for DCA. At 280 days there was no data
34 reported for TCA but for DCA ~ 2-fold decrease in labeling of control. At 350 days there was
35 no data for DCA but a reported ~ 2.3 fold decrease in labeling of control with TCA.   The
36 authors reported that the increases at day 14 for TCA and DCA exposure and the decrease at day
37 28 for DCA exposure were statistically significant although a small number of animals were
38 examined. Thus, although there may be some uncertainty in the exact magnitude of change,
39 there was at most ~5-fold of control labeling for DCA within after 14 days of exposure that was

followed by a decrease in DNA synthesis by day 28 of treatment. These data show that hepatocytes undergoing DNA synthesis represented a small population of hepatocytes with the highest level with either treatment less than 1% of hepatocytes. Rates of cell division were reported to be less than control for both DCA and TCA by 40 and 52 weeks of treatment.

In this study the authors reported that there was no necrosis with the 2.0 g/L DCA dose for 52 weeks and conclude that necrosis is a recurring but inconsistent result with chronic DCA treatment. Histological examination of the livers involved in the present study found little or no evidence of such damage or overt cytotoxicity. It was assumed that this effect has little bearing on data on replication rates. Foci and tumors were combined in reported results and therefore cannot be compared the results Bull et al (2002) or to DeAngelo et al. (1999). Prevalence rates were not reported. Data were reported in terms of "lesions" with DCA-induced "lesions" containing a number of smaller lesions that were heterogeneous and more eosinophilic with larger "lesions" tending to less numerous and more basophilic. For TCA results using this paradigm, the "lesions" were reported to be less numerous, more basophilic, and larger than those induced by DCA. The DCA-induced larger "lesions" were reported to be more "uniformly reactive to c-Jun and c-Fos but many nuclei within the lesions displaying little reactivity to c-Jun. The authors stated that while most DCA-induced "lesions" were homogeneously immunoreactive to c-Jen and C-Fos (28/41 lesions), the rest were stained heterogeneously. For TCA-induced lesions, the authors reported not difference in staining between "lesions" and normal hepatocytes in TCA-treated animals. Again, of note is that not only were "lesions" comprised of foci and tumors at different stages of progression reported in these results, but that also DCA and TCA results were reported for different durations of exposure.

2.3.2.9. Pereira 1996

The focus of this study was to report the dose-response relationship for the carcinogenic activity of DCA and TCA in female B6C3F1 mice and the characteristics of the lesions. Female B6C3F1 mice (7-8 weeks of age) were given drinking water with either DCA or TCA at 2.0, 6.67, or 20 mmol/L and neutralized with sodium hydroxide to a pH or 6.5-7.5. The control received 20 mmol/L sodium chloride. Conversion of mmol/L to g/L was as follows: 20.0 mmol/L DCA = 2.58 g/L, 6.67 mmol/L DCA = 0.86 g/L, 2.0 mmol/L = 0.26 g/L, 20.0 mmol/L TCA = 3.27 g/L, 6.67 mmol/L TCA = 1.10 g/L, 2.0 mmol/L TCA = 0.33 g/L. The concentrations were reported to be chosen so that the high concentration was comparable to those previously used by us to demonstrate carcinogenic activity. The mice were exposed till sacrifice at 360 (51 weeks), or 576 days (82 weeks) of exposure. Whole liver was reported to be cut into ~ 3 mm blocks and along with representative section of the visible lesions fixed and embedded in paraffin and stained with H&E for histopathological evaluation of foci of altered hepatocytes, hepatocellular adenomas, and hepatocellular carcinomas. The slides were reported to be evaluated blind. Foci of altered hepatocytes in this study were defined as containing 6 or more cells and hepatocellular adenomas were distinguished from foci by the occurrence of compression at greater than 80% of the border of the lesion.

1   Body weights were reported to be decreased only the highest dose of DCA from 40
2   weeks of treatment onward. For TCA there were only 2 examination periods (weeks 51 and 82)
3   that had significantly different body weights from control and only at the highest dose.
4   Liver/body weight percentage was reported in comparison to concentration graphically and
5   shows a dose-response for DCA with steeper slope than that of TCA at 360 and 576 days of
6   exposure. The authors report that all three concentrations of DCA resulted in increased
7   vacuolation of hepatocytes.(probably due to glycogen removal from tissue processing). Using a
8   score of 1 – 3, (with 0 indicating the absence of vacuolization, +1 indicating vacuolated
9   hepatocytes in the periportal zone, + 2 indicating distribution of vacuolated hepatocytes in the
10   midzone, and +3 indicating maximum vacuolization of hepatocytes throughout the liver), the
11   authors also reported "the extent of vacuolization of the hepatocytes in the mice administered 0,
12   2.0, 6.67 or 20.0 mmol/l DCA was scored as 0.0, 0.80 ± 0.08, 2.32 ± 0.11, or 2.95 ± 0.05,
13   respectively."
14
15   Cell proliferation was reported to be determined in treatment groups containing 10 mice
16   each and exposed to either DCA or TCA for 5, 12, or 33 days with animals implanted with
17   miniosmotic pumps 5 days prior to sacrifice and administered BrdU. Tissues were
18   immunohistochemically stained for BrdU incorporation. At least 2000 hepatocytes/mouse were
19   reported to be evaluated for BrdU-labeled and unlabeled nuclei and the BrDU-labeling index was
20   calculated as the percentage of hepatocytes with labeled nuclei. Pereira (1996) reported a dose-
21   related increase in BrDU labeling in 2000 hepatocytes that was statistically significant at 6.67
22   and 20.mmol/L DCA at 5 days of treatment but that labeling at all exposure concentrations
23   decreased to control levels by day 12 and 33 of treatment. The largest increase in BrdU labeling
24   was reported to be a 2-fold of controls at the highest concentration of DCA after 5 days of
25   exposure. For TCA all doses (2.0, 6.67 and 20 mmol/L) gave a similar and statistically
26   significant increase in BrDU labeling by 5 days of treatment (~ 3-fold of controls) but by days 12
27   and 33 there were no increases above control values at any exposure level. Given the low level
28   of hepatocyte DNA synthesis in quiescent control liver, these results indicate a small number of
29   hepatocytes underwent increased DNA synthesis after DCA or TCA treatment and that by 12
30   days of treatment these levels were similar to control levels in female B6C3F1 mice.
31
32   Incidence of foci and tumors in mice administered DCA or TCA (prevalence or number
33   of animals with tumors of those examined at sacrifice) in this report are given below:
34
35   *Prevalence:*
36
37

Treatment at 82 weeks	N	Foci number	%	adenomas number	%	carcinomas number	%
20.0 mmol NACL	90	10	11.1	2	2.2	2	2.2
20.0 mmol DCA	19	17	89.5*	16	84.2*	5	26.3*
6.67 mmol DCA	28	11	39.3*	7	25.0*	1	3.6
2.0 mmol DCA	50	7	14.0	3	6.0	0	0

Treatment	N						
20.0 mmol TCA	18	11	61.1*	7	38.9*	5	27.8%*
6.67 mmol TCA	27	9	33.3*	3	11.1	5	18.5*
2.0 mmol TCA	53	10	18.9	4	7.6	0	0

*p <0.05

Treatment at
51 weeks

Treatment	N						
20.0 mmol NACL	40	0	0	1	2.5	0	0
20.0 mmol DCA	20	8	40.0*	7	35*	1	5
6.67 mmol DCA	20	1	5	3	15	0	0
2.0 mmol DCA	40	0	0	0	0	0	0
20.0 mmol TCA	20	0	0	2	15.8	5	25*
6.67 mmol TCA	19	0	0	3	7.5	0	0
2.0 mmol TCA	40	3	7.5	3	2.5	0	0

Multiplicity:

Treatment at 82 weeks	N	foci/mouse	adenomas/mouse	carcinomas/mouse
20.0 mmol NACL	90	0.11 ± 0.03	0.02 ± 0.02	0.02 ± 0.02
20.0 mmol DCA	19	7.95 ± 2.00**	5.58 ± 1.14**	0.37 ± 0.17*
6.67 mmol DCA	28	0.39 ± 0.11*	0.32 ± 0.13*	0.04 ± 0.04
2.0 mmol DCA	50	0.14 ± 0.05	0.06 ± 0.03	0
20.0 mmol TCA	18	1.33 ± 0.31**	0.61 ± 0.22*	0.39 ± 0.16*
6.67 mmol TCA	27	0.41 ± 0.13*	0.11 ± 0.06	0.22 ± 0.10*
2.0 mmol TCA	53	0.26 ± 0.08	0.08 ± 0.04	0

*p <0.05 ** p<0.01

Treatment at 51 weeks	N	Foci/mouse	Adenoma/mouse	Carcinoma/mouse
20.0 mmol NACL	40	0	0.03 ± 0.03	0
20.0 mmol DCA	20	0.60 ± 0.22**	0.45 ± 0.17**	0.10 ± 0.10
6.67 mmol DCA	20	0.05 ± 0.05	0.20 ± 0.12	0
2.0 mmol DCA	40	0	0	0
20.0 mmol TCA	20	0	0.15 ± 0.11	0.50 ± 0.18*
6.67 mmol TCA	19	0	0.21 ± 0.12	0
2.0 mmol TCA	40	0.08 ± 0.04	0.08 ± 0.04	0

1   These data show the decreased power of using fewer than 50 mice, especially at shorter
2   durations of exposure. By 82 weeks of exposure increased adenoma and carcinomas induced by
3   TCA or DCA treatment are readily apparent.
4
5   The foci of altered hepatocytes and the tumors obtained from this study were reported to
6   be basophilic, eosinophilic or mixed containing both characteristics. DCA was reported to
7   induce a predominance of eosinophilic foci and tumors, with over 80% of the foci and 90% of
8   the tumors in the 6.67 and 20.0 mmol/L concentration groups being eosinophilic. Only
9   approximately half of the lesions were characterized as eosinophilic with the rest being
10   basophilic in the group administered 2.0 mmol/L DCA. The eosinophilic foci and tumors were
11   reported to consistently stained immunohistochemically for the presence of GST-π, while
12   basophilic lesions did not stain for GST-π, except for a few scattered cells or small areas
13   comprising less than 10% of foci. The foci of altered hepatocytes in the TCA treatment groups
14   were approximately equally distributed between basophilic and eosinophilic in tincture.
15   However, the tumors were predominantly basophilic lacking GST-π (21 of 28 or 75%) including
16   all 11 hepatocellular carcinomas. The limited numbers of lesions, i.e., 14, in the sodium chloride
17   (vehicle control) group were characterized as 64.3, 28.6, and 7.1% basophilic, eosinophilic, and
18   mixed, respectively.
19
20   *Phenotype:*
21
22   Treatment   N   %Foci
23   at 51 and 82 weeks   basophilic   eosinophilic   mixed

Treatment at 51 and 82 weeks	N	basophilic	eosinophilic	mixed
20.0 mmol NACL	10	70.0	30.0	0
20.0 mmol DCA	150	3.3	96.7	0
6.67 DCA	11	18.2	81.8	0
2.0 mmol DCA	7	42.8	57.2	0
20.0 mmol TCA	22	36.4	54.6	9.1
6.67 mmol TCA	11	45.5	54.5	0
2.0 mmol TCA	13	38.5	61.5	0

Treatment at 51 and 82 weeks	N	basophilic	Tumors eosinophilic	mixed
20.0 mmol NACL	4	50.0	25.0	25.5
20.0 mmol DCA	105	2.9	96.1	1.0
6.67 DCA	10	10	90	0
2.0 mmol DCA	3	0	100	0
20.0 mmol TCA	18	61.1	22.2	16.7
6.67 mmol TCA	6	100	0	0
2.0 mmol TCA	4	100	0	0

1   These data for female B6C3F1 mice shows that DCA and TCA treatment induced a
2 mixture of basophilic or eosinophilic foci. The pooling of the data between time and adenoma
3 vs. carcinoma decreases the ability to ascertain the phenotype of tumor due to treatment or the
4 progression of phenotype with time as well as the small number of tumor examined at lower
5 exposure concentrations. Foci that occurred at 51 and 82 weeks were presented as one result.
6 Adenomas and carcinoma data were pooled as one endpoint (n= number of total foci or tumors
7 examined). Therefore evolution of phenotype between less to more malignant stages of tumor
8 were lost.
9
10 *2.3.2.10. Pereira and Phelps 1996*
11
12   The focus of this study was to determine tumor response and phenotype in MNU-treated
13 mice after DCA or TCA exposure. The concentrations of DCA or TCA were the same as Pereira
14 (1996). For Pereira (1996) the animals were reported to be 7-8 weeks of age when started on
15 treatment and sacrificed after 360 or 576 days of exposure (51 or 82 weeks). For this study and
16 Tao et al. (2004), animals were reported o be 6 weeks of age when exposed to DCA or TCA via
17 drinking water and to be 31 or 52 weeks of age at sacrifice. Thus, exposure time would be ~ 24
18 or 45 weeks. A control group of non-MNU treated animals was presented for female B6C3F1
19 mice treated for 31 or 52 weeks and are discussed below. Although this paradigm appears to be
20 the same paradigm as those reported in Pereira (1996), fewer animals were studied. The number
21 of animals in each group varied between 8 controls and 14 animals in the 2.0 mmol/l treatment
22 groups. In mice that were not treated with MNU, but were treated with either DCA or TCA at 31
23 weeks, there were no reported statistically significant treatment-related effect upon the yield of
24 foci or altered hepatocytes and liver tumors but the number of animals examined was small and
25 therefore of limited power to detect a response. The results below indicate a DCA-related
26 increase in foci and percentage of mice with foci.
27
28 Treatment till 31 weeks of age:
29

Treatment	No	Foci/mouse	incidence %	Adenomas/mouse	incidence %
20.0 mmol NACL	15	0.13 ± 0.13	6.7	0.13 ± 0.13	not reported
20.0 mmol DCA	10	0.40 ± 0.16	40%	0	0%
6.67 DCA	10	0.10 ± 0.10	10%	0	0%
2.0 mmol DCA	15	0	0	0	0
20.0 mmol TCA	10	0	0	0	0
6.67 mmol TCA	10	0	0	0	0
2.0 mmol TCA	15	0	0	0	0

39
40   See Section 4.2.3 for further discussion of the results of co-exposures to MNU and DCA
41 or TCA from this study.
42

1   *2.3.2.11. Ferreira-Gonzalez et al. 1995*

2

3   The focus of this study was the investigation of differences in H-ras mutation spectra in
4 hepatocellular carcinomas induced by TCA or DCA in male B6C3F1 mice. 28-day old mice
5 were exposed for 104 weeks to 0. 1.0 g or 3.5 g/l DCA or 4.5 g/l TCA that was pH adjusted.
6 Tumors observed from this treatment were diagnosed as either hepatocellular adenomas or
7 carcinomas. DNA was extracted from either spontaneous, DCA- or TCA-induced hepatocellular
8 carcinomas. Samples for analysis were chosen randomly in the treatment groups of which 19%
9 of untreated mice had spontaneous liver hepatocellular carcinomas (0.26 carcinomas/animal),
10 DCA treatment induced 100% prevalence at 3.5 g/l (5.06 carcinomas/animal) and 70.6%
11 carcinomas at 1.0 g/l (1.29 carcinomas/animal). TCA treatment was reported to induce 73.3%
12 prevalence at 4.5 g/l (1.5 carcinomas/animal). The number of samples analyzed was 32 for
13 spontaneous carcinomas, 33 for mice treated with 3.5 g/l DCA, 13 from mice treated with 1.0 g/
14 DCA, and 11 from mice treated with 4.5 g/l TCA. This study has the advantage of comparison
15 of tumor phenotype at the same stage of progression (hepatocellular carcinoma), for allowance of
16 the full expression of a tumor response (i.e. 104 weeks), and an adequate number of spontaneous
17 control lesions for comparison with DCA or TCA treatments. However, tumor phenotype at an
18 endstage of tumor progression reflects of tumor progression and not earlier stages of the disease
19 process.

20

21   There were no ras mutations detected except at H-61 in DNA from spontaneously arising
22 tumors of control mice. Only 4/57 samples from carcinogen-treated mice were reported to
23 demonstrate mutation other than in the second exon of H-ras. In spontaneous liver carcinomas,
24 58% were reported to show mutations in H-61 as compared with 50% of tumor from 3.5 g/l
25 DCA-treated mice and 45% of tumors from 4.5.g/l TCA-treated mice. Thus, there was a
26 heterogeneous response for this phenotypic marker for the spontaneous, DCA-, and TCA-
27 treatment induced hepatocellular carcinomas.

28

29   All samples positive for mutation in the exon 2 of H-ras were sequenced for the
30 identification of the base change responsible for the mutation. The authors noted that H-ras
31 mutations occurring in spontaneously developing hepatocellular carcinomas from B6C3F1 male
32 mice are largely confined to codon 61 and involve a change from CAA to either AAA or CGA or
33 CTA in a ratio of 4:2:1. They noted that in this study, all of the H-ras second codon mutations
34 involved a single base substitution in H-61 changing the wild-type sequence from CAA to AAA
35 (80%), CGA (20%) or CTA for the 18 hepatocellular carcinomas examined. In the 16
36 hepatocellular carcinomas from 3.5 g/l DCA treatment with mutations, 21% were AAA
37 transversions, 50% were CGA transversions, and 29% were CTA transversions. For the 6
38 hepatocellular carcinomas from 1.0 g/l DCA with mutations, 16% were an AAA transversion,
39 50% were a CGA transversion, and 34% were a CTA transversion. For the 5 hepatocellular
40 carcinomas from 4.5 g/l TCA with mutations, 80% were AAA transversions, 20% CGA
41 tranversions, and 0% were CTA transversions. The authors note that the differences in
42 frequency between DCA and TCA base substitutions did not achieve statistical significance due
43 to the relatively small number of tumors from TCA-treated mice. They note that the finding of

1   essentially equal incidence of H-ras mutations in spontaneous tumors and in tumors of
2   carcinogen-treated mice did not help in determining whether DCA and TCA acted as
3   "genotoxic" or "non-genotoxic" compounds.
4
5   *2.3.2.12. Pereira et al. 2004*
6
7   Pereira et al (2004) exposed 7-8 week old female B6C3F1 mice treated with "AIN-76A
8   diet" to neutralized 0, or 3.2 g/l DCA in the drinking water and 4.0 or 8.0 g/kg L-methionine
9   added to their diet. The final concentration of methionine in the diet was estimated to be 11.3 and
10   15.3 g/kg. Mice were sacrifice 8 and 44 weeks after exposure to DCA with body and liver
11   weights evaluated for foci, adenomas, and hepatocellular carcinomas.   No histological
12   descriptions were given by the authors other than tinctoral phenotype of foci and adenomas for a
13   subset of the data. The number of mice examined was 36 for the DCA + 8.0 g/kg methionine or
14   4.0 g/kg methionine group sacrificed at 44 weeks. However for the DCA-only treatment group
15   the number of animals examined was 32 at 44 weeks and for those groups that did not receive
16   DCA but either methionine at 8.0 or 4.0 g/kg, there were only 16 animals examined. All groups
17   examined at 8 weeks had 8 animals per group. Liver glycogen was reported to be isolated from
18   30-50 mg of whole liver. Peroxisomal acyl-CoA oxidase activity was reported to be determined
19   using lauroyl-CoA as the substrate and was considered a marker of peroxisomal proliferation.
20   Whole liver DNA methylation status was analyzed using a 5-MeC antibody.
21
22   Methionine (8.0 g/kg) and DCA co-exposure was reported to result in the death of 3 mice
23   while treatment with methionine (4.0 g/kg) and DCA or methionine (8.0g/kg) alone was reported
24   to kill one mouse in each group. The authors reported that "There was an increased in body
25   weight during weeks 12 to 36 in the mice that received 8.0 g/kg methionine without DCA. There
26   was no other treatment-related alteration in body weight." However the authors do not present
27   the data and initial or final body weights were not presented for the differing treatment groups.
28   DCA treatment was reported to increase % liver/body weight ratios at 8 and 44 weeks to about
29   the same extent (i.e., ~ 2.4 –fold of control at 8 weeks and 2.2-fold of control at 44 weeks).
30   Methionine co-exposure was reported to not affect that increase (~ 2.4-fold, 2.2-fold, and 2.1-
31   fold of control after DCA treatment alone, DCA/4 g/kg methionine, and DCA/8 mg/kg
32   methionine treatment for 8 weeks, respectively). There was a slight increase in % liver/body
33   weight ratio associated with 8.0 g/kg methionine treatment alone in comparison to controls (~
34   7%) at 8 weeks with no difference between the two groups at 44 weeks.
35
36   After 8 weeks of only DCA exposure, the amount of glycogen in the liver was reported to
37   be ~ 2.09-fold of the value for untreated mice (115 mg/g vs. 52.5 mg/g glycogen in treated vs.
38   control, respectively at 8 weeks). Both 4 g/kg and 8 g/kg methionine co-exposure reduced the
39   amount of DCA-induced glycogen increase in the liver (~ 1.64-fold of control for DCA /4.0 g/kg
40   methionine and ~ 1.54-fold of control for DCA/8.0 mg/kg methionine). Thus, for treatment with
41   DCA alone or with the two co-exposure levels of methionine, the magnitude of the increase in
42   liver weight was greater than that of the increase in liver glycogen (i.e., 2.42-fold vs. 2.09-fold of
43   control % liver/body weight vs. glycogen content for DCA alone, 2.20-fold vs. 1.64-fold of

1   control % liver/body weight vs. glycogen content for DCA/4.0 g/kg methionine, 2.10-fold vs.
2   1.54-fold of control % liver/body weight vs. glycogen content for DCA/8.0 g/kg methionine).
3   Thus, the magnitudes of treatment-related increases were higher for % liver/body weight than for
4   glycogen content in these groups. In regard to percentage of liver mass that glycogen
5   represented, the control value for this study is similar to that presented by Kato-Weinstein et al
6   (2001) in male mice (~ 60 mg glycogen per gram liver) and represents ~ 6% of liver mass.
7   Therefore a doubling of the amount of glycogen is much less than the 2-fold increases in liver
8   weight observed for DCA exposure in this paradigm. This data suggests that DCA-related
9   increases in liver weight gain are not only the result of increased glycogen accumulation, and
10   that methionine co-exposure is affecting glycogen accumulation to a much greater extent than
11   the other underlying processes that are contributing to DCA-induced hepatomegaly after 8 weeks
12   of exposure. The authors reported that 8-weeks of DCA exposure alone did not result in a
13   significant increase in cell proliferation as measured by PCN index (neither data or methods were
14   shown). This is consistent with other data showing that DCA effects on DNA synthesis were
15   transient and had subsided by 8 weeks of exposure.
16
17   The levels of lauroyl-CoA oxidase activity were reported to be increased (~ 1.33-fold of
18   control) by DCA treatment alone at 8 weeks and to be slightly reduced by 8 g/kg methionine
19   treatment alone (~ 0.83 fold of control). Methionine co-exposure was reported to have little
20   effect on DCA-induced increases in lauroyl-CoA oxidase activity. The levels of DNA
21   methylation were reported to be increased by 8.0 g/kg methionine only treatment at 8 weeks ~
22   1.32-fold of control, and reduced by DCA only treatment to ~ 0.44-fold of control. DCA and 4.0
23   g/kg methionine co-exposure gave similar results as controls (within 2%). Co-exposures of DCA
24   and 8.0 g/kg methionine treatments were reported to increase DNA methylation 1.22-fold of
25   controls after 8 weeks of co-exposure.
26
27   In the 44-week study, the authors report that foci and hepatocellular adenomas were
28   found. However, the authors do not report the incidences of these lesions in their study groups
29   (how many of the treated animals developed lesions). As noted above, the numbers of animals in
30   these groups varied widely between treatments (e.g. n= 36 for DCA and co-exposure to 8.0g/kg
31   methionine but only n= 16 for 8 g/kg methionine treatment alone). Although reporting
32   unscheduled deaths in the 8.0g/kg methionine and DCA co-exposure groups, the authors did not
33   indicate whether these mortalities occurred in the 44-week or 8-week study groups.
34   Multiplicities of foci and adenoma data were presented. DCA was reported to induce 2.42 ±
35   0.38 foci/mouse and 1.28 ± 0.31 adenomas/mouse (m ± SE) after 44 weeks of treatment. The
36   DCA-induced foci and adenomas were reported to stain as eosinophilic with "relatively large
37   hepatocytes and nuclei". The authors did not present data on the % of foci and adenomas that
38   were eosinophilic using this paradigm. The addition of 4.0 or 8.0 g/kg methionine to the AIN-
39   76A diet was reported to reduce the number of DCA-induced adenomas/mouse to 0.167 ± 0.093
40   and 0.028 ± 0.028, respectively. However, the addition of 4.0 g/kg methionine to the DCA
41   treatment was reported to increase the number of foci/mouse (3.4 ± 0.46 foci/mouse). The
42   addition of 8.0 g/kg methionine to the DCA treatment was reported to yield 0.94 ± 0.24
43   foci/mouse. There were no foci or tumors in the 16 mice that received either the control diet or

1 the 8.0 g/kg methionine treatment without DCA. The authors did not report whether methionine
2 treatment had an effect on the tincture of the foci or adenomas induced by DCA.
3
4   Therefore, a very high level of methionine supplementation to an AIN-760A diet, was
5 shown to affect the number of foci and adenomas, i.e., decrease them, after 44 weeks of co-
6 exposure to very high exposure concentration of DCA. However, a lower level of methionine
7 co-exposure increased the incidence of foci at the same concentration of DCA. Methionine
8 treatment alone at the 8 g/kg level was reported to increase liver weight, decrease lauroyl-CoA
9 activity and to increase DNA methylation. No histopathology was given by the authors to
10 describe the effects of methionine alone. Co-exposure of methionine with 3.2 g/l DCA was
11 reported to decrease by ~ 25% DCA-induced glycogen accumulation and increase mortality, but
12 not to have much of an effect on peroxisome enzyme activity (which was not elevated by more
13 than 33% over control for DCA exposure alone). The authors suggested that their data indicate
14 that methionine treatment slowed the progression of foci to tumors. Whether, these results
15 would be similar for lower concentrations of DCA and lower concentrations of methionine that
16 were administered to mice for longer durations of exposure, cannot be ascertained from this data.
17 It is possible that in a longer-term study, the number of tumors would be similar. Whether,
18 methionine treatment co-exposure had an effect on the phenotype of foci and tumors was not
19 presented by the authors in this study. Such data would have been valuable to discern if
20 methionine co-exposure at the 4.0 mg/kg level that resulted in an increase in DCA-induce foci,
21 resulted in foci of a differing phenotype or a more heterogeneous composition than DCA
22 treatment alone.
23
24 *2.3.2.13. DeAngelo et al. 2008*
25
26   In this study, neutralized TCA was administered in drinking water to male B6C3 F1 mice
27 (28-30 days old) in three studies. In the first study control animals received 2 g/l sodium
28 chloride while those in the second study were given 1.5 g/l neutralized acetic acid (HAC) to
29 account for any taste aversion to TCA dosing solutions. In a third study deionized water served
30 as the control. No differences in water uptake were reported. Mean initial weights were reported
31 to not differ between the treatment groups (19.5 ± 2.5 g $- 21.4 \pm 1.6$ g or ~ 10% difference). The
32 first study was reported to be conducted at the U.S. EPA laboratory in Cincinnati, OH in which
33 mice were exposed to 2 g/l sodium chloride, or 0.05, 0.5, or 5 g/l TCA in drinking water for 60
34 weeks. There were 5 animals at each concentration that were sacrificed at 4, 15, 31, and 45
35 weeks with 30 animals sacrificed at 60 weeks of exposure. There were 3 unscheduled deaths in
36 the 0.05 g/l TCA group leaving 27 mice at final necropsy. For the other exposure groups there
37 were 29 or 30 animals at final necropsy. In the second study, also conducted in the same
38 laboratory, mice were reported to be exposed to 1.5 g/l neutralized acetic acid or 4.5 g/l TCA for
39 104 weeks. Serial necropsies were conducted (5 animals per group) at 15, 30, and 45 weeks of
40 exposure and on, 10 animals in the control group at 60 weeks. For this study, a total of 25
41 animals were sacrificed in interim necropsies in the 1.5 g/l HAC group and 15 in the 4.5 g/l TCA
42 group. There were 7 unscheduled deaths in the HAC group and 12 in the 4.5 g/l TCA group
43 leaving 25 animals at final necropsy and 30 animals in the final necropsy groups, respectively.

Study 3 was conducted at the U.S EPA laboratory in RTP NC. Mice were exposed to deionized water or 0.05 or 0.5 g/l TCA in the drinking water for 104 weeks with serial necropsies (n=8 per group) conducted at 26, 52, and 78 weeks. There were 19-21 animals reported at interim sacrifices and 17 unscheduled deaths in the deionized water group, 24 unscheduled deaths in the 0.05 g/l TCA group, and 24 unscheduled deaths in the 0.5 g/l TCA group. This left 34 mice at final necropsy in the control group, 29 mice in the 0.05 g/l TCA group, and 27 mice in the 0.5 g/l group.

At necropsy, liver, kidneys, spleen and testes weights were reported to be taken and organs examined for gross lesions. Tissues were prepared for light microscopy and stained with H& E. At termination of the exposure periods, a complete rodent necropsy was reported to be performed. Representative blocks of tissue were examined only in 5 mice from the high dose and control group with the exception of gross lesions, liver, kidney, spleen and testis at interim and terminal sacrifices. If the number of any histopathologic lesions in a tissue was "significantly increased above that in control animals" then that tissue was reported to be examined in all TCA dose groups. For Study # 3 a second contract pathologist reviewed 10% of the described hepatic lesions. No "major differences" were reported between the two pathologic diagnoses. The prevalence and multiplicity of hepatic tumors were reported to be derived by performing a histopathologic examination of surface lesions and four sections cut from each of four tissue blocks excised from each liver lobe. Tumor prevalence was reported to be calculated as the percentage of the animals with a neoplastic lesion compared to the number of animals examined. Tumor multiplicity was reported to be calculated by dividing the number of each lesion or combined adenomas and carcinomas by the number of animals examined. Preneoplastic large foci of cellular alteration were also observed over the course of the study.

The prevalence and severity of hepatocellular cytoplasmic alterations, inflammation, and necrosis were reported to be determined using a scale based on the amount of liver involved of 1 = minimal (occupying 25%), 2 = mild (occupying 25-50%), 3 = moderate (occupying 50 -75%) and 4 = marked (occupying >75%). The only "significant change outside of the liver" was reported to be testicular degeneration. Lactate dehydrogenase (LDH) was determined in arterial blood collected at 30 and 60 weeks (study 1) and 4, 30, and 104 weeks (study 2). Cyanide insensitive palmitoyl coenzyme A oxidase (PCO) was also reported to be measured. Five days prior to sacrifice, tritiated thymidine (studies 1 and 2) or BrdU (study 3) was administered via miniosmotic pumps and the number of hepatocyte nuclei with grain counts >6 were scored in 1000 cells or chromogen pigment over nuclei (BrdU). The labeling index was calculated by dividing the number of labeled hepatocyte nuclei by total number of hepatocytes scored. Total neoplastic and preneoplastic lesions (multiplicity) were counted individually or combined (AD and CA) for each animal. The analysis of tumor prevalence data were reported to include only those animals examined at the scheduled necropsies or animals surviving to week 60 (Study 1) or longer than 78 weeks (Studies 2 and 3). The data from all the scheduled necropsies was combined for an overall test of treatment-related effect.

1   For Study #1 (60-week exposure) all TCA treated groups experienced a decrease in
2   drinking water consumption with the decreases in drinking water for the 0.5 g/l and 5 g/l TCA
3   exposure groups reported as statistically significant by the authors. The water consumption in
4   ml/kg-day was reported to be reduced by 11%, 17%, and 30% in the 0.05g/l, 0.5 g/l and 5 g/l
5   TCA treated groups compared to 2 g/l NaCl control animals as measured by time-weighted mean
6   daily water consumption measured over the study. The control value was reported to be 171
7   ml/kg/day. Although the 0.05 g/l exposure concentrations were not measured, the 0.5 g/l and 5
8   g/l solutions were within 4% of target concentrations. The authors estimated that the mean daily
9   doses were 0, 8 mg/kg, 68 mg/kg and 602 mg/kg per day. For the 102 week studies the mean
10   water consumption with deionized water was reported to be 112 ml/kg/day and 132 ml/kg/day
11   for control animals given 1.5 g/L HAC. Therefore, there appeared to be a 35% decrease in water
12   consumption between the controls in Study #1 given 2 g/l NaCl and controls in a Study # 3 given
13   deionized water but conducted at a different laboratory. There appeared to be a 23% reduction in
14   water consumption between animals given 2 g/l NaCl and those given 1.5 g/l HAC at the same
15   laboratory (Study # 2). As the concentrations of TCA were increased, there would be a
16   corresponding increase in the amount of sodium hydroxide needed to neutralize the solutions and
17   a corresponding increase in salts in the solution as well as TCA. The authors did not address nor
18   discuss the differences in drinking water consumption between the differing control solutions
19   between the studies. DeAngelo et al. (1999) reported mean drinking water consumption of 147
20   ml/kg/day in control mice of over 100 weeks and that the highest dose of DCA (3.5 g/l) reduced
21   drinking water consumption by 26%. Carter et al. (1995) reported that DCA at 5 g/l to decrease
22   drinking water consumption by 64% and 46% but 0.5 g/l DCA to not affect drinking water
23   consumption. While reporting that Study #1 showed that increasing TCA concentration
24   decreased drinking water consumption, the drinking water consumption in Studies #2 and #3
25   were similar between controls and TCA exposure groups with both being less than the control
26   and low TCA concentration values reported in Study # 1 (i.e. in Study #2 the 1.5 g/l HAC and
27   4.5 g/l TCA drinking water consumption was ~ 130 ml/kg/day and in Study # 3 the drinking
28   water consumption was ~ 112 ml/kg/day for the deionized water control and 0.05 g/l and 0.5 g/l
29   TCA exposure groups). Thus, the drinking water concentrations for Study #3 was ~ 35% less
30   than for the control values for Study #1 and was also ~ 25% less than for DeAngelo et al (1999).
31   The reasons for the apparently lower drinking water averages for Study #3 and the lack of effect
32   of the addition of 0.5 g/l TCA that was reported in Study #1 and in other studies, was not
33   discussed by the authors.
34
35   In Study #1, there was little difference between exposure groups (n=5) noted for the final
36   body weights (mean range of 27.6 – 28.1 g) in mice sacrificed after 4 weeks of exposure.
37   However, absolute liver weight and % liver/body weight ratios increased with TCA dose. The %
38   liver/body weight ratios were 5.7 ± 0.4%, 6.2 ± 0.3%, 6.6 ± 0.4%, and 7.7. ± 0.6% for the 2 g/l
39   NaCl control, 0.05 g/l, 0.5 g/l, and 5 g/l TCA exposure groups, respectively. These represent
40   1.09-fold, 1.16-fold, and 1.35-fold of control levels that were statistically significant. At 15
41   weeks of exposure the fold increases in % liver/body weight ratios were 1.14-fold, 1.16-fold,
42   1.47-fold of controls for 0.05 g/l 0.5 g/l and 5 g/l TCA. At 31 weeks of exposure the fold
43   increases in % liver/body weight ratios were 0.98-fold, 1.09-fold, 1.59-fold of controls for 0.05

1   g/l 0.5 g/l and 5 g/l TCA. At 45 weeks of exposure the fold increases in % liver/body weight
2   ratios were 1.13-fold, 1.45-fold, 1.98-fold of controls for 0.05 g/l 0.5 g/l and 5 g/l TCA. At 60
3   weeks of exposure the % liver/body weight ratios were 0.94-fold, 1.25-fold, 1.60-fold of controls
4   for 0.05 g/l 0.5 g/l and 5 g/l TCA. Thus, the range of increase at the lowest level of TCA
5   exposure (i.e., 0.05 g/l) was 0.94-fold to 1.14-fold of controls. These data consistently show
6   TCA-induced increases in liver weight from 4 to 60 weeks of the study that were dose-related.
7   For the 0.5 g/l exposure group, the magnitude of the increase compared to control was reported
8   to be about the same between weeks 4 and 30 with the highest increase reported to be at week 45
9   (1.45-fold of control). In regard to the correspondence with magnitude of difference in dose of
10 TCA and liver weight increase, there was ~ 2-fold increase in liver weight gain corresponding to
11 10-fold increases in TCA concentration at 4 weeks of exposure. For the 4 and 15-week
12 exposures there was ~ 3.3- and 3.9-fold difference in liver weight that corresponded to a 100-
13 fold difference in exposure concentration of TCA (i.e., 0.05 vs. 5.0 g/l TCA).
14
15   The small number of animals examined, n=5, limit the power of the study to determine
16 the change in % liver/body weight up to 45 weeks, especially at the lowest dose. However the
17 0.05 g/l TCA exposure groups at 4 week and 15 weeks were reported to significantly increase %
18 liver/body weight ratios. The % liver/ body weight ratios for all of the treatment groups and the
19 ability to detect significant changes were affected by changes in final body weight and changing
20 numbers of animals. After 4 to 30 weeks of exposure, the final body weights of mice increased
21 in control animals but were within 11% of each other between weeks 31 and 60. The %
22 liver/body weight ratios in controls decreased from 4 to 31 weeks and were slightly elevated by
23 60 weeks compared to the 31-week level. Although control values were changing, there
24 appeared to be no difference between control values and treated values in final body weight for
25 any duration of exposure with the exception of the 5 g/l TCA exposure group after 60 weeks of
26 exposure, which was decreased by ~ 15%. At the 31-week and 60-week exposure durations, the
27 0.05 g/l TCA groups did not have increased % liver/body weight ratios over controls.
28
29   In Study #2, conducted in the same laboratory but with a 1.5 g/l HAC solution used for
30 control groups, there was less than 5% difference in final body weights between control mice
31 give HAC and those treated with 4.5 g/l TCA up to 45 weeks. However final body weight was
32 reduced by TCA treatment by 104 weeks by ~ 15%. Between the interim sacrifices of 15, 30,
33 and 45 weeks, the % liver/body weight ratios in control mice were similar at 15 and 45 weeks (~
34 4.8%) but greater in the 30-week control group (5.3% or ~ 10 % greater than other interim
35 control groups). The TCA-induced increases in body weight were 1.60-fold, 1.40-fold, and 1.79-
36 fold of control for the 15, 30, and 45 week groups exposed to 4.5 g/l TCA in Study # 2. The
37 smaller magnitude of TCA-induced liver weight increase at 30-weeks that that for 15 and 45
38 weeks, was a reflection of the increased % liver/body weight ratio reported for the HAC control
39 at that time point.
40
41   Comparisons can be made between Study #1 and Study #2 for 4.5 g/l or 5.0 g/l TCA
42 exposure levels and controls for 15, 30/31 and 45 weeks of exposure to ascertain the consistency
43 of response from the same laboratory. Although the two studies had differing control solutions

1   and reported different drinking water consumption overall, they were exposing the TCA groups
2   to almost the same concentration of TCA in the same buffered solutions for the same periods of
3   time with the same number of mice per group.   Between Study #1 and Study #2, there were
4   consistent % liver/body weight ratios induced by either 5.0 g/l TCA and 4.5 g/l TCA at weeks 15
5   and 30/31 (i.e. within 3% of each other). The % liver/body ratios for these exposure groups
6   ranged from 7.3% - 7.7% between weeks 15 and 30/31 for the ~ 5.0 g/l TCA exposure in both
7   studies. Final body weights were within 10%. While the % liver/body weight ratios induced by
8   ~ 5.0 g/l TCA were similar, the magnitude of increase in comparison to the controls was 1.47-
9   fold and 1.59-fold of control for Study #1, and 1.60-fold and 1.40-fold of control for Study #2
10   after 15 and 30/31 weeks of exposure, respectively. At 45 weeks, the % liver/body weight ratios
11   were within 11% of each other (9.4% vs. 8.4%) and final body weights were within 2 % of each
12   for this exposure concentration between the two studies giving a 1.98-fold and 1.79-fold of
13   control % liver/body weight, respectively. Thus, the apparent magnitude of TCA-induced
14   increase in % liver/body weight was affected by control values used as the basis for comparison.
15   The % liver/body weights reported for either 4.5 g/l TCA or 5.0 g/l TCA exposure groups for
16   weeks 15 and 30/31 was similar between the two studies conducted in the same laboratory.
17
18   Study #3 was conducted in a separate laboratory, interim sacrifice times were not the
19   same as for Study # 1, the number of animals examined differed (n= 5 for Study #1 and n=8 for
20   Study #3), and control animals studied for comparative purposes were given different drinking
21   water solutions (deionized water vs. 2 g/l NaCl). Most importantly the body weights reported at
22   52 weeks was much grated than that reported at 45 weeks for Studies #1 and #2. However, a
23   comparison of TCA-induced liver weight gain and the effects of final body weight can be made
24   between the 0.05 g/l and 0.5 g/l TCA exposure groups at 30 weeks (Study #1) and 26 weeks
25   (Study #3), at 45 weeks and 60 weeks (Study # 1), and 52 weeks (Study # 3). At 31 weeks there
26   was <2% difference in mean final body weights between control and the two TCA-treatment
27   groups in Study #1. There was also little difference between the TCA-treated groups at week in
28   Study #3 at week 26 and the TCA treatment groups in at week 31 in Study # 1 (i.e., range of 42.6
29   g – 43.5 g for 0.05 and 0.5 g/l TCA treatments in Studies # 1 and #3). However in Study # 3, the
30   control value was 12% lower than that of Study #1 for mean final body weight. Based on final
31   body weights, there would be an expectation of similar results between the two studies at the 26
32   and 30 week time points. At the 45 week (Study #1), and 52-week (Study #3), and 60-week
33   (Study #1) durations of exposure, the mean final body weights varied little between their
34   corresponding control groups at each sacrifice time (less than 4% variation between control and
35   TCA-treated groups). However, there was variation in mean final body weights between the
36   differing sacrifice times. Control and TCA-treated groups were reported to have lower mean
37   final body weights at 45 weeks of exposure in Study #1 than at either 30 weeks or at 60 weeks.
38   The 45-week mean final body weights in Study #1 were also reported to be lower than those at
39   52 weeks in Study #3. Control mean body weight values were 28% higher at 52 weeks in Study
40   #3 than 45 weeks in Study #1 and 15% higher for 60 weeks in Study #1. In essence, for Study #
41   1 mean final body weights went down between 31 and 45 weeks of exposure and then went back
42   up at 60 weeks of exposure for control mice (~ 43 g, ~ 40 g, and ~ 44 g for 31, 45 and 60 weeks,
43   respectively) as well as for both TCA concentrations. However, for Study #3 final mean body

1 weights went up between 26 and 52 weeks of exposure for control mice (~ 39 g vs. ~51 g) and
2 for both TCA concentrations. While for Study # 1 the % liver/body weight ratios were 0.98-fold
3 and 1.09-fold of control at 31 weeks of exposure, at week 45 the ratios were 1.13-fold and 1.45-
4 fold of control, and at week 60 they were 0.94-fold and 1.25-fold of controls for the 0.05 g/l and
5 0.5 g/l TCA exposure levels, respectively. For Study # 3, the pattern differed than that of Study
6 # 1. There was a 1.07-fold and 1.18-fold of control % liver/body weight for 26 weeks but a 0.92-
7 fold and 1.04-fold of control % liver/body weight change at 52 weeks of exposure at 0.05 g/l and
8 0.5 g/l TCA exposure, respectively.
9
10 Thus, there appeared to be differences in control and the treatment groups at the 26 week
11 sacrifice groups in Study # 3 that was not apparent at the 52-week sacrifice time. Overall, the
12 final body weights appeared to be similar between controls and TCA treatment groups at the 52-
13 week sacrifice time in Study # 3 and at the 31-, 45-, and 60-week sacrifice times in Study #1.
14 However, although consistent within sacrifice times, the final body weights differed between the
15 various sacrifice times in Studies # 1 and # 3. The patterns of % liver/body weight at differing
16 and similar sacrifice times appeared to differ between the Study # 1 and Study #3 at the same
17 concentrations of TCA. The largest difference appeared to be between week 45 group in Study #
18 1 and week 52 group in Study #3 where both concentrations of TCA were reported to induce
19 increases in % liver/body weight in one study but to have little difference in the other. The
20 differences in mean final body weights between these two sacrifice times were also the largest
21 although control and TCA-treatment groups had little difference on this parameter. Similar to
22 the work of Kjellstrand et al with TCE (Kjellstrand et al., 1983a), the groups with the lower body
23 weight appeared to have the greatest response in liver weight increase.
24
25 These data illustrate the variability in findings of % liver weight induction between
26 laboratories, studies, choice of controls solutions, and the affects of final body weights on this
27 parameter. They also illustrate the limitations for determining either the magnitude or pattern of
28 liver weight increases using a small number of test animals. As animals age the size of their
29 liver changes but also during the latter parts of the lifespan, foci and spontaneously occurring
30 liver tumors can affect liver weight. The results of Study #1 show a consistent dose-response in
31 TCA liver weight increases at 4 and 15 week time periods over a range of concentration from
32 0.05 g/l to 5 g/l TCA.
33
34 In regard to non-neoplastic pathological changes the authors reported that " Increased
35 incidences and severity of centrilobular cytoplasmic alterations, inflammation, and necrosis were
36 the only nonproliferative changes seen in livers of animals exposed to TCA for 60 weeks (Tables
37 7-9; Study 1. Incidences were between 21 and 93%; severity ranged from minimal to mild; and
38 some lesions were transient. Centrilobular cytoplasmic alterations (Table 7) were the most
39 prominent nonproliferative lesion. The incidence and severity were dose related and
40 significantly increased at all TCA concentrations. Centrilobular alterations are a low-grade
41 degeneration of the hepatocytes characterized by an intense eosinophilic cytoplasm with deep
42 basophilic granularity (microsomes) and slight hepatomegaly. The distribution ranged from
43 centrilobular to diffuse. The incidence of inflammation was increased significantly in the 5 g/l

1   TCA treatment group (Table 8), but was significantly lower in the 0.05- and 0.5 g/l groups
2   between 31 and 45 weeks, but abated by 60 weeks. There was a significant dose-related trend,
3   but a significant increase in severity was only found at 5 g/l. No alteration in the severity of this
4   lesion was observed. The occurrence and severity of nonproliferative lesions in animals exposed
5   to 0.5 and 4.5 g/l TCA for 104 weeks were similar to those observed at 60 weeks (data not
6   shown). No pathology outside the liver was observed except for a significant dose-related trend
7   and incidence of testicular tubular degeneration at 0.5 and 5 g/l TCA."
8
9   The results shown in Table # 7 by the authors for the 60-week TCA-exposed mice did
10   not show a dose-response for either incidence or severity of centrilobular cytoplasmic alterations.
11   They reported a 7%, 48%, 21% and 93% incidence and a 0.10 ± 0.40, 0.70 ± 0.82, 0.34 ± 0.72
12   and 1.60 ± 0.62 mean severity score for control, 0.05, 0.5, and 5.0 g/l TCA exposure groups,
13   respectively. Thus, for control, 0.05 and 0.5 g/l TCA exposure there was less than minimal (i.e.,
14   score of 1 or occupying less than 25% of the microscopic field) severity of this finding for the 27
15   to 30 mice examined in each group. Only slight hepatomegaly is noted by the authors to be
16   included in their description of the centrilobular cytoplasmic alteration. Interestingly, the
17   elevation of this parameter for both incidence and severity in the 0.05 g/l TCA exposed group
18   compared to 0.5 g/l exposure group did not correspond to an increase in % liver/body weight for
19   this same exposure group. While the % liver/body weight ratio was 32% higher, the incidence
20   and severity of this lesion were reported to be half that in the 0.5 g/l vs. 0.05 g/l exposure groups
21   after 60 days of TCA exposure. Thus, TCA-induced hepatomegaly did not appear to be
22   associated with this centrilobular cytoplasmic change. Similarly the incidence of hepatic
23   inflammation was reported to be 10%, 0%, 7%, and 24% and severity, 0.11 ± 0.40, 0.09 ± 0.30,
24   0.12 ± 0.33, and 0.29 ± 0.48 for control, 0.05, 0.5, and 5.0 g/l TCA exposure groups,
25   respectively. Thus, at no TCA exposure concentration was the incidence more than 24% and the
26   severity was considerably less than minimal. The reported results for hepatic necrosis were
27   pooled from data from the 5 mice exposed for either 30 or 45 weeks (n= 10 total). No incidences
28   of necrosis were reported for either control or 0.05 g/l TCA exposed mice. At 0.5 g/l TCA 3/10
29   mice were reported to have necrosis but at a severity level of 0.50 ± 0.97. At 5.0 g/l TCA 5/10
30   mice were reported to have necrosis but at a severity level of 1.30 ± 1.49.   The limitations of the
31   small number of animals pooled in this data are obvious. However, there does not appear to be
32   much more than minimal necrosis at the highest dose of TCA between 30 and 45 weeks and this
33   response is reported by the authors to be transient.
34
35   Serum LDH activity was reported by the authors for 31 and 60 week TCA exposures in
36   Study #1. They state that "There was a dose-related trend at 31 weeks; serum LDH was
37   significantly increased at 0.5 and 5 g/l TCA (161 ± 39 and 190 ± 44, respectively vs. 100 ± 28 IU
38   for the control). LDH activity returned to control levels at 60 weeks. Similarly, elevated LDH
39   levels were observed at early time periods for 0.5 and 4.5 g/L TCA during the 104 week
40   exposure (data not shown: Studies 2 and 3)." The data presented by the author for Study #1 are
41   from 5 animals/group for the 30-week results and 30 animals/group for the 60-week results.   Of
42   interest is for the 60-week data, there appears to be 50% decreased in LDH activity at 0.05 and ~
43   25% decrease in LDH activity at 0.5 g/l TCA treatment with the LDH level reported to be the

1   same as control for the 5 g/l TCA exposure group. For the 31-week data, in which only 5
2   animals were tested in each treatment group, there appeared to be a slight increase at the 0.5 g/l
3   (60% increase over control) and 5 g/l (90% increase over control) treatment groups. The data for
4   necrosis detected by light microscopy and by LDH level is consistent with no changes from
5   control detected at the 0.05 g/l TCA treatment group and less than minimal necrosis of on a 60%
6   increase in LDH level over control reported for 0.5 g/l TCA treatment.   Even at the highest dose
7   of 5.0 g/l TCA there is still little necrosis or LDH release reported over control.
8
9   Data for testicular tubular degeneration was reported for Study # 1 after 60-weeks of
10   TCA exposure. The incidence of testicular tubular degeneration was reported to be 7%, 0%,
11   14%, and 21% for mice exposed to 2.0 g/l NaCl, 0.05 g/l, 0.5 g/l and 5.0 g/l TCA. The severity
12   of the lesions was reported to be 0.10 ± 0.40, 0, 0.17 ± 0.47, and 0.21 ± 0.41 with a significant
13   trend with dose reported by the authors for severity and for the 0.5 g/l and 5 g/l treatment groups
14   to be significantly increased over control incidence levels. Of note, similar to the % liver/body
15   weight ratios and hepatic inflammation values for this data set, the values for testicular tubular
16   degeneration were slightly higher in control mice than 0.05 g/l TCA exposed mice. In regard to
17   mean severity levels for testicular degeneration, although still minimal, there was little difference
18   between the results for reported for the 0.5 g/l TCA and 5.0 g/l TCA exposed mice.
19
20   In regard to peroxisome proliferation, liver PCO activity was presented for up to 60
21   weeks (Study #1) and 104 weeks (Study #2). Similar to the data for LDH activity, ~ 30 animals
22   were examined at the 60-week time point but only 5 animals per exposure group were examined
23   for 4-, 15-, 31-, and 45-week results. The data are presented in a figure and in some instances
24   hard to determine the magnitude of change. Similar to other reports, the baseline level of PCO
25   activity was variable between control groups and ranged 2.7-fold (~ 1.49 to 4.06 nmol NAD
26   reduced/min/mg protein given by the authors). There appeared to be little change in PCO
27   activity between the 0.05 g/l TCA exposure and control levels for up to 45 weeks of exposure
28   (i.e., the groups with n= 5) in Study # 1. For the 60-week group the 0.05g/l TCA group PCO
29   activity was ~ 1.7-fold of control but was not statistically significant.   For the 0.5 g/l TCA
30   treatment groups, the increase ranged from ~1.3-fold to 2.7-fold of control after 4-, 15-, 31-, and
31   45-weeks of exposure with the largest differences reported at 4 and 60 weeks (i.e., 2.2-fold and
32   2.7-fold of control, respectively).   For the 5.0 g/l TCA exposure groups, the increase ranged
33   from ~ 3.2-fold to ~ 5.7-fold of control after 4, 15, 31, and 45 weeks of exposure. While the
34   data at 60-weeks had the most animals examined (~ 30 vs. 5) with ~ 1.7-fold, 2.7-fold, and 4.5-
35   fold of control PCO activity, at this time period the authors report the occurrence of tumors had
36   already occurred. At the earlier time points of 4 and 15 weeks, there was a difference in the
37   magnitude TCA-induced increase in PCO activity. As displayed graphically, at 4 weeks the
38   PCO increase was ~ 1.3-fold, 2.4-fold, and 5.3-fold of control for 0.05, 0.5, and 5.0 g/l TCA,
39   respectively, while at 15 weeks, the PCO levels were decreased by 5%, increased to 1.3-fold, and
40   increased to 3.2-fold of control with only the 5.0 g/l treatment group difference to be statistically
41   significant.
42

1   For Study # 2 the authors present a figure (figure #4) that states that PCO values were
2   given for mice given HAC or 4.5 g/l TCA for 4-60 weeks. However the data presented in Figure
3   #4 appears to be for 15-, 30-, 45- and 104-week exposures. The number of mice is not given in
4   the figure but the methods section states that serial section were conducted on 5 mice/group for
5   these interim sacrifice periods. The number of mice examined for PCO activity at 104 weeks
6   was not given by the authors but the number of mice at final sacrifice was given as 25. The
7   levels of PCO in the control tissues varied by ~ 33% for weeks 15 to 45 but there was a ~ 5-fold
8   difference between the level reported at 104 weeks and that for the earlier time periods in control
9   mice shown in the figures (~ 2.23 vs. 0.41 nmol NAD reduced/min/mg protein as given by the
10   authors). The increase over control induced by 4.5 g/l TCA in Study # 2 was shown to be ~ 6.9-
11   fold, 4.8-fold, 3.6-fold, and 19-fold of controls for 15, 30, 45 and 104 weeks, respectively.
12
13   Therefore, at a comparable level of TCA exposure (~5.0 g/l), number of mice examined
14   (n=5), and durations of exposure (15, 30, and 45 weeks), the increase in PCO activity induced by
15   ~ 5.0 g/l TCA varied between 3.2- to 5.7-fold of control in Study #1 and between 3.6- to 6.9-fold
16   of control in Study #2. There was not a consistent pattern between the two studies in regard to
17   level of PCO induction from ~ 5 g/l TCA and duration of exposure. The lowest TCA-induced
18   PCO activity increase was recorded at 15 weeks in Study #1 (i.e., 3.2-fold of control) and highest
19   PCO activity increase was recorded at 15 weeks in Study #2 (i.e., 6.9-fold of control). No PCO
20   data was reported for data in Study #3 with the exception of the authors stating that "PCO
21   activity was significantly elevated for the 0.5 g/l TCA exposure over the 104 weeks (study 3).
22   The extent of the increases was similar to those measured for 0.5 g/l TCA (200-375%: data not
23   shown) in Study 1." No other details are given for PCO activity in Study #3.
24
25   Hepatocyte proliferation was reported by the authors to be assessed by either
26   incorporation of tritiated thymidine (Studies #1 and #2) or BrdU (Study # 3) into hepatocyte
27   nuclei. As noted previously, these techniques measure DNA synthesis and not necessarily
28   hepatocyte proliferation. The authors did not report if specific areas of the liver were analyzed
29   by autoradiographs or how many autoradiographs were examined in the analyses they conducted.
30   For later time points of examination (60 -104 weeks) the authors did not indicate whether
31   hepatocytes in foci or adenomas were excluded from DNA synthesis reports. The authors
32   present data for what are clearly, 31, 45, and 60 week exposure for Study # 1 as the percent
33   tritiated thymidine labeled nuclei. An early time point that appears to be 8 weeks is also given.
34   However for Study # 1 only 4 week and 15 week durations were tested so it cannot be
35   established what time period the earlier time point represents. What is very apparent from the
36   data presented for Study # 1 is that the baseline level of tritiated thymidine incorporation was
37   relatively high and highly variable for the 5 animals examined (~ 8% of hepatocytes were
38   labeled). There did not appear to be an apparent pattern of TCA treatment groups at this
39   timepoint with the 0.05 and 5.0 g/l TCA groups having a similar percentage of labeled
40   hepatocytes and for 0.5 g/l TCA reported to have a 60% reduction in labeled hepatocytes. After
41   31 weeks of exposure the control values were reported to be 2% of hepatocytes labeled. The
42   authors report that only the 5.0 g/l TCA group had a statistically significant increase of control
43   and was elevated to ~ 6% of hepatocytes. The two lower doses of TCA had similar reported

1 incidences of labeled hepatocytes of 4.5% that were not reported to be statistically significant.
2 For the 45-week exposure period in Study #1, the control value was reported to be 1.2% with
3 only the 5.0 g/l TCA value reported to be statistically significantly increased at 3.2% and the
4 other two TCA groups to be similar to control. Finally, for the 60 week group from Study #1,
5 the control value was reported to be 0.6% of hepatocytes labeled and the only the 0.5 g/l TCA
6 dose reported to be statistically significantly increased over control at 3.2%. What is clear from
7 this study is that the control value for the unidentified early time point is much higher than the
8 other values. There should not be such a large difference in mature mice nor such a high level.
9 The difference in control values between the earlier time point and the 31-week time point was
10 4-fold. The difference between the earlier time point and the 45-week time point was ~ 7-fold.
11 There did not appear to be an increase in hepatocyte tritiated thymidine labeling due to any
12 concentration of TCA at the early unidentified time point (~ week 10 from the figure) from
13 Study # 1. There was no dose-response apparent for the other study periods and the % of
14 hepatocytes labeled were 3% or less. These results indicated DNA synthesis was not increased
15 by 10-60 week exposures to TCA exposure that induced increased liver tumor response.
16
17   For Study # 2 results were reported for tritiated thymidine incorporation into hepatocytes
18 in a figure that was labeled as 4.5 g/l TCA and control tissue for 104 weeks but showed data for
19 15, 30, and 45 weeks of exposure. Of note is that the control values for this study were much
20 lower than that reported for Study #1. The % of hepatocytes labeled with tritiated thymidine was
21 reported to be ~ 2% for the 15 week exposure period and less than 1% for the 30- and 45-week
22 exposure periods. For the 4.5 g/l TCA exposures the % hepatocytes labeled with tritiated
23 thymidine were ~ 2-4% at all time points with only the 45 week period identified by the authors
24 as statistically significant.
25
26   For Study # 3, rather than tritiated thymidine, BrdU was used as a measure of DNA
27 synthesis. The results are presented in Figure # 8 of the report in which the 0.5 g/l TCA
28 concentration is mislabeled as 0 g/l and the Figure is mislabeled as having a duration of 104
29 weeks but the data is presented for 26, 52, and 78 weeks of exposure. The % of hepatocytes at
30 26 weeks was reported to be ~ 1-2% for the control, 0.05 and 0.5 g/l TCA groups. At 52 weeks
31 the control value was ~ 1% the 0.05 g/l TCA value was less than 0.1% and the 0.5 g/l TCA value
32 was ~ 3.5% but not statistically significant. At 78 weeks of exposure the control value was
33 reported to be ~ 0.2% with only the 0.05 g/l TCA group having a statistically significant increase
34 over control.
35
36   From this data, the estimated control values for DNA synthesis at similar time points of
37 exposure ranged from 0.4% to 2% at 26-31 weeks and ~0.1 to 1.2% at 45-52 weeks.   The results
38 for Study #1 and # 2 were inconsistent in regard to the magnitude of tritiated thymidine
39 incorporation but consistent in that there was a lot of variability in these measurements, not a
40 consistent pattern with time that was TCA–dose related, and ,even at the highest dose of TCA,
41 did not indicate much of an increase in cell proliferation 15-45 weeks of exposure. Similarly the
42 results for Studies #1 and #3 indicate that the two lower doses of TCA there were not generally

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1 statistically significant increases in DNA synthesis from 15-45 weeks of exposure although there
2 was an increase in liver tumor response at later time points.
3
4   The authors reported that "all gross and microscopic histopathological alterations were
5 consistent across the three studies."   However, the histological descriptions that follow were
6 focused on the liver for both neoplastic and non-neoplastic parameters. As stated above, only a
7 few animals (n= 5) from the control and high TCA dose level were examined for lesions other
8 than liver, kidneys, spleen and testes. Thus, whether other neoplastic lesions were induced by
9 TCA exposure cannot be determined from this set of studies.
10
11   Study # 1 was conducted for 60 weeks. Although of short duration and using 30 or less
12 animals, the authors reported in the text that "a significant trend with dose was found for liver
13 cancer. The prevalence and multiplicity of adenomas (38%; 0.55 ± 0.15) or carcinoma (38%;
14 0.42 ± 0.11) were statistically significant at 602 mg/kg/day TCA compared to control (7%; 0.07
15 ± 0.05) [sic for both adenoma and carcinoma the same value was given, mean $\pm$ SD]. When
16 either an adenoma or a carcinoma was present, statistical significant was seen at both 5 g/l (55%;
17 1.00 ± 0.19) and 0.5 g/l (38%: 0.52 ± 0.14 TCA exposure groups compared to control (13%; 0.13
18 ± 0.06). No significant change in liver neoplasia were reported to be observed by the authors at
19 0.05 g/l TCA. Preneoplastic large foci of cellular alteration (24%) were seen in the 5 g/l TCA
20 control compared to control."
21
22   Although not statically significant, there was an incidence of 15% adenoma in the 0.05
23 g/l TCA treatment group (n= 27) and a multiplicity of 0.15 ± 0.07 adenomas/mouse reported
24 with both values being twice that of the values given for the controls (n= 30). The incidence and
25 multiplicity for carcinomas was approximately the same for the 0.05 g/l TCA treatment group
26 and the control group. Given the small number of animals examined, the study was limited in its
27 ability to determine statistical significance for the lower TCA exposure level. The fold increases
28 of incidence and multiplicity of adenomas at 60 weeks was 2.1-fold, 3.0-fold, 5.4-fold of control
29 incidence and 2.1-fold, 3.4-fold, and 7.9-fold of control multiplicity for 0.05 g/l, 0.5 g/l, and 5 g/l
30 exposure to TCA. For multiplicity of adenomas and carcinomas combined there was a 1.46-fold,
31 4.0-fold, and 7.68-fold of control values. Analysis of tumor prevalence data for this study
32 included only animals examined at scheduled necropsy. Since most animals survived till 60
33 weeks, most were included and a consistent time point for tumor incidence was reported.
34
35   There are significant discrepancies for reporting of data for tumor incidences in this
36 report for the 104 week data. While the methods section and Table describing the dose
37 calculation and animal survival indicate that Study #3 control animals were administered
38 deionized water and those from Study# 2 were given HAC, Table 6 of the report gives 2 g/l NaCl
39 as the control solution given for Study #2 and 1.5g/l HAC for Study #3. A comparison of the
40 descriptions of animal survival and tumor incidence and multiplicity between the results given in
41 DeAngelo et al. (2008) and George et al, (2000) shows not only that the control data presented in
42 DeAngelo et al (2008) for Study #3 to be the same data as that presented by George et al (2000)
43 previously, but also indicates that rather than 1.5 g/l HAC, the tumor data presented in DeAngelo

1 et al (2008) is for mice exposed to deionized water. DeAngelo et al (2008) did not report that
2 this data was from a previous publication.
3

Descriptor	George et al., 2000	DeAngelo et al., 2008
Species	mouse	mouse
Strain	B6C3F1	B6C3F1
Gender	Male	Male
Age	28-30 days	28-30 days
Source	Charles River, Portage	Charles River, Portage
Mean Initial body wt	19.5 ± 2.5 g	19.5 ± 2.5 g
Water consumption	111.7 ml/kg/day	112 ml/kg/day
Laboratory	RTP NC	RTP NC
# Animals at start	72	72
# Animals at interim sac.	22	21
# Unscheduled deaths	16	17
# Animals at final sacrifice	34	34
# Animals for pathology	65	63
Adenoma incidence	21.4 %	21%
Adenoma multiplicity	0.21 ± 0.06	0.21 ± 0.06
Carcinoma incidence	54.8%	55%
Carcinoma multiplicity	0.74 ± 0.12	0.74 ± 0.12

23   For Studies # 2 and # 3 tumor prevalence data was reported in the methods section of the
24 report to include necropsies of animals that survived greater than 78 weeks and thus included
25 animals that were scheduled for necropsy but also those which were moribund and sacrificed at
26 differing times. Thus, for the longer times of study, there was a mixture of exposure durations
27 that included animals that were ill and sacrificed early and those that survived to the end of the
28 study. Animals that were allowed to live for longer periods or who did not die before scheduled
29 sacrifice times had a greater opportunity to develop tumors. However, animals that died early
30 may have died from tumor-related causes. The mislabeling of the tumor data in DeAngelo et al
31 (2008) has effects on the interpretation of results for if the tumor results table was not mislabeled
32 it would indicated 17 animals were included in the liver tumor analysis that were not included in
33 the final necropsy and that the 7 unscheduled deaths could not account for the total number of
34 "extra" mice included in the tumor analysis so some of the animals had to have come from
35 interim sacrifice times (78 weeks or less) and that for Study #3 the data from 9 animals at
36 terminal sacrifice were not used in the tumor analysis. Not only was the control data mislabeled
37 for Study #3, but the control data was also apparently mislabeled for Study #2 as being 2.0 g/l
38 NaCl rather than 1.5 g/l HAC. Of the 42 animals used for the tumor analysis in Study #3, only
39 34 were reported to have survived to interim sacrifice so that 8 animals were included from
40 unscheduled deaths. However, the authors report that there were 17 unscheduled deaths in the
41 study not all were included in the tumor analysis. The basis for the selection of the 8 animals for
42 tumor analysis was not give by the authors.
43

1   Not only are the numbers of control animals used in the tumor analysis different between
2 two studies (25 mice in Study #2 and 42 mice in Study #3), but the liver tumor results reported
3 for Study #2 and Study #3 were very different. Of the 42 "control" mice examined from Study
4 #3, the incidence and multiplicity of adenomas was reported to be 21% and 0.21 ± 0.06,
5 respectively. For carcinomas, the incidence and multiplicity was reported to be 55% and 0.74 ±
6 0.12, respectively, and for the incidence and multiplicity of adenomas and carcinomas combined
7 reported to be 64% and 0.93 ± 0.12, respectively. For the 25 mice reported by the authors for
8 Study # 2 to have been treated with "2.0g/l NaCl" but were probably exposed to 1.5 g/l HAC, the
9 incidence and multiplicity of adenomas was 0%. For carcinomas, the incidence and multiplicity
10 was reported to be 12% and 0.20 ± 0.12, respectively and for the incidence and multiplicity of
11 adenomas and carcinomas combined to be 12% and 0.20 ± 0.12, respectively. Therefore while ~
12 64% the 42 control mice in Study #3 were reported to have adenomas and carcinomas, only 12%
13 of the 25 mice were reported to have adenomas and carcinomas in Study #2 for 104-weeks.
14
15   While the effect of using fewer mice in one study vs. the other will be to reduce the
16 power of the study to detect a response, there are additional factors that raise questions regarding
17 the tumor results.   Not only were the tumor incidences were reported to be higher in control
18 mice from Study # 3 than Study #2, but the number of unscheduled deaths was reported to also
19 be 2-fold higher. The age, gender, and strain of mouse were reported to be the same between
20 Study #2 and #3 with only the vehicles differing and weight of the mice to be reported to be
21 different.   Although the study by George et al (2000) describes the same control data set as for
22 Study #3 as being for animals given deionized water, there is uncertainty as to the identity of the
23 vehicle used for the tumor results reported for Study #3 and there are some discrepancies in
24 reporting between the two studies. As discussed below in Section 2.5, the differences in the
25 weight of the mice between Studies #1, #2 and #3 is critical to the issue of differences in
26 background tumor rate and hence interpretability of the study.
27
28   As noted by Leakey et al. (2003b), the greatest correlation with liver tumor incidence and
29 body weight appears between the ages of 20 and 60 weeks in male mice. As reported in Section
30 2.5., the mean 45-week body weight reported for control male B6C3F1 mice in the George et al
31 (2000) study, which is the same control data as DeAngelo et al (2008) was ~ 50 g. This is a
32 much greater body weight than reported for Study # 1 at 45 weeks (i.e., 39.6 g) and for Study #
33 2 at 45 weeks (i.e., 39.4 g). Using probability curves presented by Leakey et al (2003b), the
34 large background rate of 64% of combined adenomas and carcinomas for Study # 3 is in the
35 range predicted for such a large body weight (i.e. ~ 65%). Such a high background incidence
36 compromises a 2-year bioassay as it prevents demonstration of a positive dose-response
37 relationship. Thus, Study # 3 of DeAngelo et al (2008) is not comparable to the results in Study
38 # 1 and # 2 for the determination of the dose-response for TCA.
39
40   The accurate determination of the background liver tumor rate is very important in
41 determining a treatment related effect. The very large background level of tumor incidence
42 reported for Study # 3 makes the detection of a TCA-related change in tumor incidence at low
43 exposure levels very difficult to determine. Issues also arise as to what the source of the tumor

1   data was in the TCA-treatment and control groups in Study #3. While 29 mice exposed to
2   0.05g/l TCA were reported to have been examined at terminal sacrifice, 35 mice were used for
3   liver tumor analysis. Similarly, while 27 mice exposed to 0.5 g/l TCA were reported to have
4   been examined at terminal sacrifice, 37 mice were used for tumor analysis. Finally, for the 42
5   control animals examined for tumor pathology in the control group, 34 were examined at
6   terminal sacrifice. Clearly more animals were included in the analyses of tumor incidence and
7   multiplicity than were sacrificed at the end of the experiment. What effect differential addition
8   of the results from mice not sacrificed at 104 weeks and the selection bias that may have resulted
9   from their inclusion on these results cannot be determined. Not only were the background levels
10   of tumors reported to be increased in the control animals in Study #3 compared to Study #2 at
11   104 weeks, but the rate of unscheduled deaths was doubled. This is also an expected
12   consequence of using much larger mice (Leakey et al 2003b).
13
14   For the 35 mice examined after 0.05 g/l TCA in Study # 3, the incidence and multiplicity
15   of adenomas was reported to be 23% and 0.34 ± 0.12, respectively. For carcinomas, the
16   incidence and multiplicity was reported to be 40% and 0.71 ± 0.19, respectively, and for the
17   incidence and multiplicity of adenomas and carcinomas combined reported to be 57% and $1.11 \pm$
18   0.21, respectively. For the 37 mice examined after 0.5 g/l TCA in Study # 3, the incidence and
19   multiplicity of adenomas was reported to be 51% and 0.78 ± 0.15, respectively. For carcinomas,
20   the incidence and multiplicity was reported to be 78% and 1.46 ± 0.21, respectively, and for the
21   incidence and multiplicity of adenomas and carcinomas combined reported to be 87% and $2.14 \pm$
22   0.26, respectively. Thus, at 0.5 g/l TCA the results presented for this study for the "104 week"
23   liver tumor data were significantly increased over the reported control values   However, these
24   results are identical to those reported in Study #3 for a 10-fold higher concentration of TCA (4.5
25   g/l TCA) for the same 104 weeks of exposure but in the much larger mice. Of the 36 animals
26   exposed to 4.5 g/l TCA in Study #2 and included in the tumor analysis, 30 animals were reported
27   to be examined at 104 weeks. The incidence and multiplicity of adenomas was reported to be
28   59% and 0.61 ± 0.16, respectively. For carcinomas, the incidence and multiplicity was reported
29   to be 78% and 1.50 ± 0.22, respectively, and for the incidence and multiplicity of adenomas and
30   carcinomas combined reported to be 89% and 2.11 ± 0.25, respectively.
31
32   The importance of selection and determination of the control values for comparative
33   purposes of tumor induction are obvious from this data. The very large difference in control
34   values between Study #2 and Study #3 is the determinant of the magnitude of the dose response
35   for TCA after 104 weeks of exposure. The tumor response for 0.5 g/l and 4.5 g/l TCA exposure
36   between the two experiments was identical. Therefore only the background tumor rate
37   determined the magnitude of the response to treatment. If a similar control values (i.e., a
38   historical control value) were used in these experiments, there would appear to be no difference
39   in TCA-tumor response between 0.5 and 4.5 g/l TCA at 104 weeks of exposure. DeAngelo et al
40   (1999) report for male B6C3F1 mice exposed only water for 79 to 100 weeks the incidence of
41   carcinomas to be 26% and multiplicity to be 0.28 lesions/mouse. For 100-week data, the
42   incidence and prevalence of adenomas was reported to be 10% and 0.12 ± 0.05 and for
43   carcinomas to be 26% and 0.28 ± 0.07. Issues with reporting for that study have already been

1   discussed in Section 2.3.2.5. However, the data for DeAngelo et al. (1999) are more consistent
2   with the control data for "1.5 g/l HAC" for Study #2 in which there were 0% adenomas and 12%
3   carcinomas with a multiplicity of 0.20 ± 0.12, than for the control data for Study #3 in which
4   64% of the control mice were reported to have adenomas and carcinomas and the multiplicity
5   was 0.93 ± 0.12. If either the control data from DeAngelo et al. (1999) or Study #2 were used
6   for comparative purposes for the TCA-treatment results of Study #2 or #3, there would be a
7   dose-response between 0.05 and 0.5 g/l TCA but no difference between 0.5 g/l TCA and 4.5 g/l
8   TCA after 100 weeks of exposure. The tumor incidence would have peaked at ~ 90% in the 0.5
9   and 4.5 g/l TCA exposure groups. These results would be more consistent with the 60-week
10   results in Study #1 in which 0.5 and 5 g/l TCA exposure groups already had incidences of 38%
11   and 55% of adenomas and carcinomas combined, respectively, compared to the 13% control
12   level. With increased time of exposure the differences between the two highest TCA exposure
13   concentrations may diminish as tumor progression is allowed to proceed further. However, the
14   use of the larger and more tumor prone mice in Study #3 also increases the tumor incidence at
15   the longer period of study.
16
17   The authors also presented data for multiplicity of combined adenomas or carcinomas for
18   mice sacrificed at weeks 26, 52, and 78 for Study #3 (n= 8 per group). No indication of
19   variability of response, incidence data, statistical significance, or data for adenomas vs.
20   carcinomas, or the incidence of adenomas was reported. The authors reported that "neoplastic
21   lesions were first found in the control and 0.05 g/l TCA groups at 52 weeks. At 78 weeks,
22   adenomas or carcinomas were found in all groups (0.29, 0.20, and 0.57 tumors/animals for
23   control, 0.05 g/l TCA, and 0.5 g/l TCA, respectively)." Because no other data were presented at
24   the 52 and 78 week time points in this study, these results cannot be compared to those presented
25   for Study #1 which was conducted for 60 weeks. Of note, the results presented from Study #1
26   for 60 weeks of exposure to control, 0.05 g/l or 0.5 g/l TCA exposure in 27-30 mice show a 13%,
27   15% and 38% incidence of hepatocellular adenomas and carcinomas and a multiplicity of $0.13 \pm$
28   0.06, 0.19 ± 0.09, and 0.52 ± 0.14, respectively. Both the incidence and multiplicity of adenomas
29   were 2-fold higher in the 0.05 g/l TCA treatment group than for the control. However, the
30   interim data presented by the authors from Study #3 for 52 weeks of exposure in only 8 mice per
31   group gives a higher multiplicity of adenomas and carcinomas for control animals (~0.25) than
32   for either 0.05 g/l TCA or 0.5 g/l TCA treatments. Again, comparisons between Study #2 and #3
33   are difficult due to difference in mouse weight.
34
35   Of note, there are no descriptions given in this report in regard to the phenotype of the
36   tumors induced by TCA or for the liver tumors reported to occur spontaneously in control mice.
37   Such information would have been of value as this study reports results for a range of TCA
38   concentration and for 60 and 100 weeks of exposure. Insight could have been gained as to the
39   effects of differing concentrations of TCA exposure, whether TCA-induced liver tumors had a
40   similar phenotype as those occurring spontaneously, as well as information in regard to effects
41   on tumor progression and heterogeneity.
42

1   Although only examining tissues from 5 mice from the control and high-dose groups only
2   at 104 weeks at organ sites other than the liver, the authors report that "neoplastic lesions at 104
3   weeks (Studies #2 and #3) at organ sites other than the liver were found in the lung, spleen,
4   lymph nodes, duodenum (lymphosarcoma), seminal vesicles, skin, and thoracic cavity of control
5   and treated animals. All were considered spontaneous for the male B6C3F1 mouse and did not
6   exceed the tumor incidences when compared to a historical control database (Haseman et. al.,
7   1984; NIEHS, 1998)." No data was shown. The limitations involved in examining only 5
8   animals in the control and high-dose groups, and the need to examine the concurrent control data
9   in each experiment, especially given the large variation in liver tumor response between long-
10   term studies carried out in the two different laboratories used for Study #2 and Study #3 using
11   the same strain and gender of mouse, make assertions regarding extrahepatic carcinogenicity of
12   TCA from this study impossible to support.
13
14   A key issue raised from this study is whether changes in any of the parameters measured
15   in interim sacrifice periods before the appearance of liver tumors (i.e., 4- 15 weeks)
16   corresponded to the induction of liver tumors. The first obstacle for determining such a
17   relationship is the experimental design of these studies in which only a full range of TCA
18   concentrations is treated for 60 weeks of exposure with a small number of animals available for
19   determination of a carcinogenic response (i.e., 30 animals or less in Study #1) and a very small
20   number of animals (n=5 group) examined for other parameters. Also as stated above, PCO
21   activity was highly variable between controls and between treatment groups (e.g., the PCO
22   activity for Study # 1 and #2 at ~ 5 g/l exposure for 15 weeks). On the other hand, most of the
23   animals that were examined at terminal sacrifice were also utilized for the tumor results without
24   the differential deletion or addition of "extra" animals for the tumor analysis. For the 60-week
25   data in Study # 1 there appeared to be a consistent dose-related increase in the incidence and
26   multiplicity of tumors after TCA exposure. The TCA-induced increases in liver tumor responses
27   can be compared with both increased liver weight and PCO activity that were also reported to be
28   increased with TCA dose as earlier events. Although the limitations of determining the exact
29   magnitude of responses has already been discussed, as shown below, the incidence and
30   multiplicity of adenomas show a dose-related increase at 60 weeks. However, the magnitude of
31   differences in TCA concentrations was not similar to the magnitude of increased liver tumor
32   induction by TCA after 60 weeks of exposure.
33
34   First of all, the greater occurrence of TCA-induced increases in adenomas than
35   carcinomas reported after 60 weeks of exposure would be expected for this abbreviated duration
36   of exposure as they would be expected to occur earlier than carcinomas. For adenoma induction,
37   there was a ~ 2-fold increase between the 0.05 g/l dose of TCA and the control group for
38   incidence (7% vs. 15%) and multiplicity (0.07 vs. 0.15 tumors/animals). However, an additional
39   10-fold increase in TCA dose (0.5 g/l) only resulted in a reported 1.8-fold greater incidence
40   (15% vs. 21%) and 2.2-fold increase in multiplicity (0.15 vs. 0.24 tumors/animal) of control
41   adenoma levels. An additional 10-fold increase in dose (5.0g/l vs. 0.5 g/l TCA) resulted in a 2.2-
42   fold increase in incidence (21% vs. 38%) and 2.9-fold increase in multiplicity (0.24 vs. 0.55
43   tumors/animal) of control adenoma levels. Thus, a 100-fold difference in TCA exposure

1 concentration resulted in differences of 4-fold of control incidence and 6-fold of control
2 multiplicity for adenomas. For adenomas or carcinomas combined (a parameter that included
3 carcinomas for which only the two highest exposure levels of TCA were reported to increase
4 incidence and multiplicity) the incidences were reported to be 13%, 15% 38%, and 55%, and the
5 multiplicity reported to be 0.13, 0.19, 0.52, and 1.00 for control, 0.05, 0.5, and 5.0 g/l TCA at 60
6 weeks. For multiplicity of adenomas or carcinomas, the 0.05 g/l TCA exposure induced a 1.5-
7 fold increase over control. An additional 10-fold increase in TCA (0.5 g/l) induced a 6-fold
8 increase in tumors/animal. An additional 10-fold increase in TCA (5.0 g/l vs. 0.5 g/l) induced an
9 additional 2.2-fold increase in tumors/animal. Therefore, using combinations of adenomas or
10 carcinomas, there was a 13-fold increase in multiplicity that corresponded with a 100-fold
11 increase in dose.
12
13   The results for adenoma induction at 60 weeks of TCA exposure (i.e., ~ 2-fold increased
14 incidences and 2- to 3-fold increases in multiplicity with 10-fold increases in TCA dose) are
15 similar to the ~ 2-fold increase in liver weight gain resulting from 10-fold differences in dose
16 reported at 4-weeks of exposure.   For PCO activity there was a ~ 30% increase in PCO activity
17 from control at 0.05 g/l TCA. A 10-fold increase in TCA exposure concentration (0.5 g/l)
18 resulted in an additional ~ 5-fold increase in PCO activity. However another 10-fold increase in
19 TCA concentration (0.5 vs. 5 g/l) resulted in a 3-fold increase in PCO activity. The 100-fold
20 increase in TCA dose (0.05 vs. 5 g/l TCA) was correlated with a 14-fold increase in PCO
21 activity.   For 15 weeks of TCA exposure there was no difference in 0.05 and control PCO
22 activity and only a 30% difference between the 0.05 and 0.5 g/l TCA exposures. There was a 7-
23 fold difference in PCO activity between the 0.5 and 5.0 g/l TCA exposure concentrations. The
24 increases in PCO activity and liver weight data at 15-weeks did not fit the magnitude of increases
25 in tumor multiplicity or incidence data at 60 weeks as well as did the 4-week data. However, the
26 TCA-induced increase in tumors at 60 weeks (especially adenomas) seemed to correlate more
27 closely with the magnitude of liver weight increase than for PCO activity at both 4 and 15 weeks.
28
29   In regard to Studies #1 and #2 there are consistent periods of study for % liver/body
30 weight with the consistency of the control values being a large factor in the magnitude of TCA-
31 induced liver weight increases. As discussed above, there were differences in the magnitude of
32 % liver/body weight increase at the same concentration between the two studies (e.g., a 1.47-fold
33 of control % liver/body weight in the 5 g/l TCA exposed group in Study #1 and 1.60-fold of
34 control in Study #2 at 15 weeks). For the two studies that had extended durations of exposure
35 (Studies #2 and #3) the earliest time period for comparison of % liver/body weight is 26 weeks
36 (Study #3) and 30 weeks (Study #2). If those data sets (26 weeks for Study # 3 and 30 weeks for
37 Study # 2) are combined, 0.05, 05, and 4.5 g/l TCA gives a % liver body/weight increase of 1.07-
38 fold, 1.18-fold and 1.40-fold over concurrent control levels. Using this parameter, there appears
39 to be a generally consistent pattern as that reported for Study #1 at weeks 4 and 15. Generally, a
40 10-fold increase in TCA exposure concentration resulted in ~ 2.5-fold increased in additional
41 liver weight observed at ~ 30 weeks of exposure which correlated more closely with adenoma
42 induction at 60 weeks than did changes in PCO activity. A similar comparison between Studies

1   of longer duration (Studies #2 and #3) could not be made for PCO activity as data were not
2   reported for Study #3.
3
4   For 104-week studies of TCA-tumor induction (Studies #2 and #3) the lower TCA
5   exposure levels (0.05 and 0.5 g/l TCA) were assayed in a separate experiment and by a separate
6   laboratory than the high dose (5.0 g/l TCA) and most importantly in larger more tumor prone
7   mice. The total lack of similarity in background levels of tumors in Study #2 and #3, the
8   differences in the number of animals included in the tumor analyses, and the low number of
9   animals examined in the tumor analysis at 104 weeks (less than 30 for the TCA treatment
10 groups) makes the determination of a dose-response TCA-induced liver tumor formation after
11 104-weeks of exposure problematic. The correlation of % liver/body weight increases with
12 incidence and multiplicity of liver tumors in Study #1 and the similarity of dose-response for
13 early induction of % liver/body weight gain between Study #1 suggest that there should be a
14 similarity in tumor response. However, as noted above, the 104-week studies had very
15 difference background rates of spontaneous tumors reported in the control mice between Study#
16 2 and #3.
17
18   The table below shows the incidence and multiplicity data for Studies #2 and #3 along
19 with the control data for DeAngelo et al. (1999) for the same paradigm. It also provides an
20 estimate of the magnitude of increase in liver tumor induction by TCA treatments if the control
21 values from the DeAngelo et al. (1999) data set were used as the background tumor rate. As
22 shown below, the background rates for Study #2 are more consistent with those of DeAngelo et
23 al (1999). Whereas there was a 2:1 ratio of multiplicity for adenomas and adenomas and
24 carcinomas between 0.5 and 5.0 g/l TCA after 60 weeks of exposure, there was no difference in
25 any of the data (i.e., adenoma, carcinoma, and combinations of adenoma and carcinoma
26 incidence and multiplicity) for these exposure levels in Study #2 and #3 for 104 weeks. The
27 difference in the incidences and multiplicities for all tumors was 2-fold between the 0.05 and 0.5
28 g/l TCA exposure groups in Study #2.   These results are consistent with the two highest
29 exposure levels reaching a plateau of response with a long enough duration of exposure (~ 90%
30 of animals having liver tumors) and with the 2-fold difference in liver tumor induction between
31 concentrations of TCA that differed by 10-fold, reported in Study #1.
32
33   If either the control values for Study #2 or the control values from DeAngelo et al (1999)
34 were used for as the background rate of spontaneous liver tumor formation, the magnitude of
35 liver tumor induction by the 0.05 g/l TCA over control levels differs dramatically from that
36 reported as control tumor rates in Study #3. To put the 64% incidence data for carcinomas and
37 adenomas reported in DeAngelo et al. (2008) for the control group of study #3 in context, other
38 studies cited in this review for B6C3F1 mice show a much lower incidence in liver tumors in
39 that: (1) the NCI (1976) study of TCE reports a colony control level of 6.5% for vehicle and
40 7.1% incidence of hepatocellular carcinomas for untreated male B6C3F1 mice (n= 70 – 77) at 78
41 weeks, (2) Herren-Freund et al (1987) report a 9% incidence of adenomas in control male
42 B6C3F1 mice with a multiplicity of 0.09 ± 0.06 and no carcinomas (n= 22) at 61 weeks, (3)
43 NTP (1990) report an incidence of 14.6% adenomas and 16.6% carcinomas in male B6C3F1

1   mice after 103 weeks (n= 48), and (4) Maltoni et al (1986) report that B6C3F1 male mice from
2   the "NCI source" had a 1.1% incidence of "hepatoma" (carcinomas and adenomas) and those
3   from "Charles River Co." had a 18.9% incidence of "hepatoma" during the entire lifetime of the
4   mice (n= 90 per group).   The importance of examining an adequate number of control or treated
5   animals before confidence can be placed in those results in illustrated by Anna et al (1994) in
6   which at 76 weeks 3/10 control male B6C3F1 mice that were untreated and 2/10 control animals
7   given corn oil were reported to have adenomas but from 76 to 134 weeks, 4/32 mice were
8   reported to have adenomas (multiplicity of 0.13 ± 0.06) and 4/32 mice were reported to have
9   carcinomas (multiplicity of 0.12 ± 0.06).
10
11   Using concurrent control values reported in Study #3, there is no increase in incidence of
12   multiplicity of adenomas and carcinomas for the 0.05g/l exposure group. However, compared to
13   either the control data from DeAngelo et al. (1999) or the control data from Study #3, there is a ~
14   2-3-fold or ~ 5-fold increased in incidence or multiplicity of liver tumors, respectively. Thus,
15   trying to determine a correspondence with either liver weight increases or increases in PCO
16   activity at earlier time points will be depend on the confidence placed in the concurrent control
17   data reported in Study #3 in the 104 week studies. As noted previously, the use of larger tumor
18   prone mice in Study #3 limits its usefulness to determine the dose-response for TCA.
19
20   The authors provide a regression analysis for "tumors/animal" or multiplicity as a percent
21   of control values and PCO activity for the 60-week and 104-week data. Whether adenomas and
22   carcinomas combined or individual tumor type were used was not stated. Also comparing PCO
23   activity at the end of the experiments, when there was already a significant tumor response rather
24   than at earlier time points, may not be useful as an indicator of PCO activity as a key event in
25   tumorigenesis. A regression analysis of this data is difficult to interpret because of the dose
26   spacing of these experiments as the control and 5 g/l exposure levels will basically determine the
27   shape of the dose response curve. The 0.05 g/l and 0.5 g/l exposure groups in the regression
28   were so close to the control value in comparison to the 5 g/l exposure, that the dose response will
29   appear linear between control and the 5.0 g/l value with the two lowest doses not affectly
30   changing the slope of the line (i.e., "leveraging" the regression). The value of this analysis is
31   limited by (1) the use of tumor prone larger mice in Study #3 that had large background rates of
32   tumors which make inappropriate the apparent combination of results from study #2 and #3 for
33   the multiplicity as percentages of control values (2) the low and varying number of animals
34   analyzed for PCO values and the variability in PCO control values (3) the appropriateness of
35   using PCO values from later time points, and (4) the dose-spacing of the experiment.
36
37   Similarly, the authors report a regression analysis that compares "percent of
38   hepatocellular neoplasia" which again is indicated by tumor multiplicity with TCA dose as
39   represented by mg/kg/day. This regression analysis also is of limited value for the same reasons
40   as that for PCO with added uncertainty as the exposure concentrations in drinking water have
41   been converted to an internal dose and each study gave different levels of drinking water with
42   one study showing a reduction of drinking water at the 5 g/l level. The authors attempt to
43   identify a NOEL for tumorigenicity using tumor multiplicity and TCA dose. However, it is not

1   an appropriate descriptor for these data, especially given that "statistical significance" of the
2   tumor response is the determinant of the conclusions regarding a dose in which there is no TCA-
3   induced effect. Only the 60-week experiment (i.e., study # 1) is useful for the determination of
4   tumor dose-response due to the issues related to appropriateness of control in study #3. A power
5   calculation of the 60-week study shows that the type II error, which should be > 50% and thus
6   greater than the chances of "flipping a coin", was 41% and 71% for incidence and 7% and 15%
7   for multiplicity of adenomas for the 0.05 g/l and 0.5 g/l TCA exposure groups. For the
8   combination of adenomas and carcinomas, the power was 8% and 92% for incidence and 6% and
9   56% for multiplicity at 0.05 g/l and 0.5 g/l TCA exposure. Therefore, the designed experiment
10   could accept a false null hypothesis, especially in terms of tumor multiplicity, at the lower
11   exposure doses and erroneously conclude that there is no response due to TCA treatment.

TCA-induced increases in liver tumor occurrence and other parameter over control after 60 weeks (Study #1)

Dose TCA g/l	Adenomas Incidence	Adenomas Multiplicity	Adenomas or carcinomas Incidence	Multiplicity	% liver/body weight 4-week	15-week	PCO activity 4-week	15-week
NaCl 7%	0.07	13%	0.13					
0.05	15% (2.1-fold)	0.15 (2.1-fold)	15% (1.2-fold)	0.19(1.5-fold)	1.09-fold	1.14-fold	1.3-fold	1.0 -fold
0.5	21% (3.0-fold)	0.24 (3.4-fold)	38% (2.9-fold)	0.52 (4.0-fold)	1.16-fold	1.16-fold	2.4-fold	1.3-fold
5.0	38% (5.4-fold)	0.55 (7.9-fold)	55% (4.2-fold)	1.00 (7.7-fold)	1.35-fold	1.47-fold	5.3-fold	3.2-fold

TCA-induced increases in liver tumor occurrence after 104 weeks (Studies # 2and #3)

Dose TCA	Adenomas Incidence	Multiplicity	Carcinomas Incidence	Multiplicity	Adenomas or Carcinomas Incidence	Multiplicity
Study #3						
1.5 g/l HAC (H$_2$0?)	21%	0.21	55%	0.74	64%	0.93
0.05 g/l TCA	23%	0.34	40%	0.71	57%	1.11
	(1.1-fold)	(1.6-fold)	(0.7-fold)	(1.0-fold)	(0.9-fold)	(1.2-fold)
0.5 g/l TCA	51%	0.78	78%	1.46	87%	2.14
	(2.4-fold)	(3.7-fold)	(1.4-fold)	(2.0-fold)	(1.4-fold)	(2.3-fold)
Study # 2						
2.0 g/l NaCl (HAC?)	0%	0	12%	0.20	12%	0.20
4.5 g/l TCA	59%	0.61	78%	1.50	89%	2.14
	(?)	(?)	(6.5-fold)	(7.5-fold)	(7.4-fold)	(11-fold)
DeAngelo et al. (1999)						
H$_2$O	10%	0.12	26%	0.28		
0.05g/TCA (S #3)	(2.3-fold)	(2.8-fold)	(1.5-fold)	(2.5-fold)		

1   0.5 g/l TCA (S #3)   (5.1-fold)   (6.5-fold)   (3.0-fold)   (5.2-fold)
2   5.0 g/l TCA (S #2)   (5.9-fold)   (6.5-fold)   (3.0-fold)   (5.4-fold

1   *2.3.2.14. DeAngelo et al. 1997*

2

3   The design of this study appears to be similar to that of DeAngelo et al. (2008) but to have
4   been conducted in F344 rats.   28-30 day old rats that were reported to be of similar weights were
5   exposed to 2.0 g/l NaCl, 0.05, 0.5, or 5.0 g/l TCA in drinking water for 104 weeks. There were
6   groups of animals sacrificed at 15, 30, 45 and 60 weeks (n= 6) for PCO analysis. There were 23,
7   24, 19, and 22, animals reported to be examined at terminal sacrifice at 104 weeks and 23, 24, 20,
8   and 22 animals reported to be used in the liver tumor analysis reported by the authors for the
9   control, 0.05, 0.5, and 5.0 g/l treatment groups, respectively. Complete pathological exams were
10   reported to be performed for all tissues from animals in the high dose TCA group at 104 weeks.
11   No indication is given as to whether a complete necropsy and pathological exam was performed
12   for controls at terminal sacrifice. Tritiated thymidine was reported to be administered at interim
13   sacrifices five days prior to sacrifice and to be examined with autoradiography. The 5 g/l TCA
14   treatment group was reported to have a reduction in growth to 89.3% of controls.

15

16   For water consumption TCA vs. reported to slightly decrease water consumption at all
17   doses with a 7%, 8%, and 4% decrease in water consumption reported for 0.05, 0.5 and 5.0 g/l
18   TCA, respectively. Body weight was decreased by 5.0 g/l TCA dose only through 78 weeks of
19   exposure to 89.3% of the control value. All of the % liver/body weight ratios were reported to be
20   slightly decreased (1-4%) by all of the exposure concentrations of TCA but the data shown does
21   not indicate if the liver weight data was taken at interim sacrifice times and appears to be only for
22   animals at terminal sacrifice of 104 weeks.

23

24   No data was shown for hepatocyte proliferation but the authors reported no TCA treatment
25   effects. For PCO there was a 2.3-fold difference between control values between the 15-week
26   and 104-week data. For the 0.05 and 0.5 g/l TCA treatment groups there was not a statistically
27   significant difference reported between control and treated group PCO levels. At 15 weeks the
28   PCO activity was reduced by 55%, increased to 1.02-fold, and increased 2.12-fold of control for
29   0.05, 0.5 and 5.0 g/l TCA exposures, respectively. For the 30 week exposure groups, the 0.05 and
30   0.5 g/l TCA groups were reported to have PCO levels within 5% of the control level. However,
31   for the 5.0 g/l TCA treatment groups there was ~2-fold of control PCO activity at the 15, 30, 45
32   and 60 weeks and at 104 weeks there was a 4-fold of control PCO activity. Of note is that the
33   control PCO value was lowest at 104 weeks while the TCA treatment group was similar to
34   interim values.

35

36   For analysis of liver tumors, there were 20-24 animals examined in each group. Unlike
37   the study of DeAngelo et al.(2008), it appeared that most of the animals that were sacrificed at
38   104 weeks were used in the tumor analysis without addition of "extra" animals or deletion of
39   animal data. The incidence of adenomas was reported to be 4.4%, 4.2%, 15%, and 4.6% and the
40   incidence of hepatocellular carcinomas was reported to be 0, 0, 0, and 4.6% for the control, 0.05,
41   0.5, and 5.0 g/l TCA exposure groups. The multiplicity or tumors/animal was reported to be 0.04,
42   0.08, 0.15, and 0.05 for adenomas and 0, 0, 0, and 0.05 for carcinomas for the control, 0.05, 0.5,
43   and 5.0 g/l TCA exposure groups. Although there was an increase in the incidence of adenomas

1   at 0.5 g/l and an increase in carcinomas at 5.0 g/l TCA, they were not reported to be statistically
2   significant by the authors. Neither were the increase in adenoma multiplicity at the 0.05 and 0.5
3   g/l exposures. However, using such a low number of animals per treatment group (n= 20 -24)
4   limits the ability of this study to determine a statistically significant increase in tumor response
5   and to be able to determine that there was no treatment-related effect. A power calculation of the
6   study shows that the type II error, which should be > 50% and thus greater than the chances of
7   "flipping a coin", was less than 6% for incidence and multiplicity of tumors at all exposure DCA
8   concentrations with the exception of the incidence of adenomas for 0.5 g/l treatment group
9   (58.7%). Therefore, the designed experiment could accept a false null hypothesis, especially in
10   terms of tumor multiplicity, at the lower exposure doses and erroneously conclude that there is no
11   response due to TCA treatment. Thus, while suggesting a lower response than for mice for TCA-
12   induced liver tumors, the study is inconclusive for determination of whether TCA induces a
13   carcinogenic response in the liver of rats.   The experimental design is such that extrahepatic
14   carcinogenicity of TCA in the male rat cannot be determined.
15
16   *2.3.2.15. DeAngelo et al., 1996.*
17
18   In this study, 28-day-old male F344 rats were given drinking water containing DCA at
19   concentrations of 0, 0.05, 0.5, or 5.0 g/l with another group was provided water containing 2.0 g/l
20   NaCl for 100 weeks. This experiment modified its exposure protocol due to toxicity (peripheral
21   neuropathy) such that the 5.0 g/l group was lowered to 2.5 g/l at 9 weeks and then 2.0 g/l at 23
22   weeks and finally to 1.0 g/l at 52 weeks. When the neuropathy did not reverse or diminish, the
23   animals were sacrificed at 60 weeks and excluded from the results. Based on measured water
24   intake in the 0, 0.05 and 0.5 g/L groups, the time-weighted average doses were reported to be 0,
25   3.6, and 40.2 mg/kg-day respectively. This experiment was conducted at an EPA laboratory in
26   Cincinnati and the controls for this group were given 2.0 g/l NaCl (study #1). In a second study
27   rats were given either deionized water or 2.5 g/l DCA which was also lowered to 1.5 g/l at 8
28   weeks and to 1.0 g/l at 26 weeks of exposure (study #2).
29
30   Although 23 animals were reported to be sacrificed at terminal sacrifice that had been
31   given 2 g/l NaCl, the number of animals reported to be examined in this group for hepatocellular
32   lesions was 3. The incidence data for this group for adenomas was 4.4% so this is obviously a
33   typographical error. The number of rats included in the water controls for tumor analysis was
34   reported to be 33 which was the same number as those at final sacrifice. The number of animals
35   at final sacrifice was reported to be 23 for 2 g/l NaCl, 21 for 0.05 g/l DCA, 23 for 0.5g/l DCA in
36   experiment #1 and 33 for deionized water and 28 for the initial dose of 2.5 g/l DCA in experiment
37   #2. Although these were of the same strain, the initial body weight was 59.1 g vs. 76 g for the 2.0
38   g/l control group vs. deionized water group. The treatment groups in both studies were similar to
39   the deionized water group. The % liver/body weights were greater (4.4% vs. 3.7% in the NaCl
40   vs. deionized water control groups (~ 20%). The number of unscheduled deaths was greater in
41   study # 2 (22%) than in study # 1 (12%).   Interim sacrifice periods were conducted.
42

1   As with the DeAngelo et al. (2008) study in mice, the number of animals reported at final
2   sacrifice was not the same as the number examined for liver tumors in study # 1 (5 more animals
3   examined than sacrificed at the 0.05 g/l DCA and 6 more animals examined than sacrificed at the
4   0.5 g/l DCA exposure groups) with n= 23, n= 26, and n= 29 for the 2g/l NaCl, 0.05 g/l DCA and
5   0.5 g/l DCA groups utilized in the tumor analysis . For study #2 the same number of rats was
6   reported to be sacrificed as examined. The source of the extra animals for tumor analysis in study
7   #1, whether from interim sacrifice or unscheduled deaths, was not given by the authors and is
8   unknown. Carcinomas prevalence data was not reported for the control group or 0.05 g/l DCA
9   group in study #1 and multiplicity data was not reported to the control group, or 0.05 g/l DCA
10   group. Multiplicity was not reported for adenomas in the 0.05 g/l DCA group in Study # 1.
11
12   There was a lack of hepatocyte DNA synthesis and necrosis reported at any dose group
13   carried out to final sacrifice at 100 weeks. The authors reported that the incidence of adenomas to
14   be 4.4% in 2 g/l NaCl control, 0 in 0.05 g/l DCA, and 17.2% in the 0.5 g/l DCA exposure groups.
15   For carcinomas no data was reported for the control or 0.05 g/l DCA group but an incidence of
16   10.3% was reported for the 0.5 g/l DCA group. The authors reported increased hepatocellular
17   adenomas and carcinomas in male F344 rats although not data was reported for carcinomas in the
18   control and 0.05 g/l exposure groups. They reported that for 0.5 g/l DCA 24.1 % vs. 4.4%
19   adenomas and carcinomas combined (Study # 1) and 28.6 vs. 3.0% (study #2) at what was
20   initially 2.5 g/l DCA but continuously reduced). Tumor multiplicity was significantly was
21   reported to be increased in the 0.5 g/l DCA group (0.04 adenomas and carcinomas/animal in
22   control vs. 0.31 in 0.5 g/l DCA in study #1 and 0.03 in control vs. 0.36 in what was initially 2.5
23   g/l DCA in study # 2). The issues of use of a small number of animals, additional animals for
24   tumor analysis in study # 1, and most of all the lack of a consistent dose for the 2.5 g/l animals in
25   study # 2, are obvious limitations for establishment of a dose-response for DCA in rats.
26
27   *2.3.2.16. Richmond et al., 1995*
28
29   This study was conducted by the same authors as DeAngelo et al. (1996) and appears to
30   report results for the same data set for the 2 g/l NaCl control, 0.05 g/l DCA and 0.5 g/l DCA
31   exposed groups. Of note is that while DeAngelo et al (1996) refer to the 28-day old rats as
32   "weanlings" the same aged rats are referred to as "adults" in this study. Male Fischer 344 rats
33   were administered time-weighted average concentrations of 0, 0.05, 0.5, or 2.4 g/l DCA in
34   drinking water. Concentrations were kept constant but due to hind-limb paralysis all 2.4 g/l DCA
35   exposed rats had been sacrificed by 60 weeks of exposure. In the 104-week sacrifice time, there
36   were 23 rats reported to be analyzed for incidence of hepatocellular adenomas and carcinomas in
37   the control group, 26 rats in the 0.05 g/l DCA group and 29 rats in the 0.5 g/l DCA exposed
38   group. This is the same number of animals included in the tumor analysis reported in DeAngelo et
39   al. (1996). Tumor multiplicity was not given. Richmond et al (1995) reported that there was a
40   4% incidence of adenomas reported in the 2.0 g/l NaCl control animals, 0% at 0.05 g/l DCA, and
41   21% in the 0.5 DCA group at 104 weeks. These figures are similar to those reported by
42   DeAngelo et al (1996) for the same data set with the exception of a 17.2% incidence of adenomas
43   reported for the 0.5 g/l DCA group. There were no hepatocellular carcinomas reported in the

1   control or 0.05 g/l exposure groups but a 10% incidence reported in the 0.5 g/l DCA exposure
2   group at 104 weeks of exposure. While carcinomas were not reported by DeAngelo et al (1996)
3   for the control and 0.05 g/l groups they are assumed to be zero in the summary data for
4   carcinomas and adenomas combined. The 10% incidence at 0.5 g/l DCA is similar to the 10.4%
5   incidence reported for this group by DeAngelo et al (1996). At 60 weeks at 2.4 g/l DCA, the
6   incidence of hepatocellular adenoma was reported to be 26% and hepatocellular carcinoma to be
7   4%. This is not similar to the values reported by DeAngelo for 2.5 g/l DCA that was
8   continuously decreased so that the estimated final concentration was 1.6 g/l DCA for 100 weeks.
9   for those animals, the incidence of adenomas was reported by DeAngelo et al (1996) to be 10.7%
10 and carcinomas 21.4%, probably more a reflect of longer exposure time allowing for adenoma to
11 carcinoma progression. The authors did not report any of the results of DCA-induced increases of
12 adenomas and carcinomas to be statistically significant. As it appears the same data set was used
13 for the 2.g/l NaCl control, 0.05 g/l DCA and 0.5 g/l DCA exposure groups as was reported in
14 DeAngelo et al (1996), the same issues arise as regarding the differences in numbers of animals
15 were included in tumor analysis than were reported to have been present at final sacrifice. As
16 stated previously for the DeAngelo et al., (1997) study of TCA in rats, the use of small numbers
17 of rats limits the detection of and ability to determine whether there was no treatment-related
18 effects, especially at the low concentrations of DCA exposure.
19
20 ## 2.4. Summaries and Comparisons Between TCE, DCA, and TCA Studies
21
22   There are a number of studies to TCE that have reported effects on the liver. However,
23 the study of this compound is difficult as its concentration does not remain stable in drinking
24 water, some studies have been carried out using TCE with small quantities of a carcinogenic
25 stabilizing agent, some studies have been carried out in whole body inhalation chambers that
26 resulted in additional oral administration and for which individual animal data were not recorded
27 throughout the experiment, and the results of gavage studies have been limited by gavage related
28 deaths and vehicle effects. In addition some studies have been conducted using the i.p route of
29 administration which results in route-related toxicity and inflammation. For many studies, liver
30 effects consisted of measured increases in liver weight with little or no description of attendant
31 histological changes induced by TCE treatment. A number of studies were conducted at a few
32 relatively high doses with attendant effects on body weight, indicative of systemic toxicity and
33 affecting TCE-induced liver weight gain. Although, many studies have been performed in male
34 mice, the inhalation studies of Kjellstrand et al. indicate that male mice, regardless of strain
35 appear to have a greater variability in response, as measured by TCE-induced liver weight gain,
36 and susceptibility to TCE-induced decreases in body weight than female mice. However, the
37 body of the TCE literature is consistent in identifying the liver as a target of TCE-induced affects
38 and with the most commonly reported change to be a dose-related TCE-induced increase in liver
39 weight in multiple species, strains, and genders from both inhalation and oral routes of exposure.
40
41   The following sections will not only summarize results for studies of TCE reported in
42 Sections 2.1 – 2.2, but provide comparison of studies of either TCA or DCA that have used
43 similar paradigms or investigated similar parameters described in Sections 2.3.1 and 2.3.2. A

1   synopsis of the results from studies of chloral hydrate and it comparison with TCE results is
2   presented in the next section (Section 2.3.5). While the study of Bull et al. (2002), described in
3   Section 2.2.21, presents data for combinations of DCA or TCA exposure for comparisons of
4   tumor phenotype with those induced by TCE, the examination of co-exposure studies of TCE
5   metabolites in rodents that are also exposed to a number of other carcinogens, and descriptions of
6   the toxicity data for brominated haloacetates that also occur with TCE in the environment, are
7   presented in Section 4.

2.4.1. Summary of Results For Short-term Effects of TCE.

11   In regard to early changes in DNA synthesis, the data for TCE is very limited   The study
12   by Mirsalis et al. (1989) used an *in vivo-in vitro* hepatocyte DNA repair and S-phase DNA
13   synthesis in primary hepatocytes from male Fischer-344 rats (180-300g) and male and female
14   B6C3F1 mice (20-29 g for male mice and 18-25 g female mice) administered TCE by gavage in
15   corn oil. They reported negative results 2-12 hours after treatment from 50-1000 mg/kg TCE in
16   rats and mice (male and female) for unscheduled DNA synthesis and repair using 3 animals per
17   group. After 24 and 48 hours of 200 or 1000 mg/kg TCE in male mice (n= 3) and after 48 hours
18   of 200 (n= 3) or 1000 (n= 4) mg/kg TCE in female mice, similar values of 0.30 to 0.69% of
19   hepatocytes were reported as undergoing DNA synthesis in those hepatocytes in primary culture
20   with only the 1000 mg/kg TCE dose in male mice at 48 hours giving a result considered to be
21   positive (~ 2.2%). No statistical analyses were performed on these measurements which were
22   obviously limited by both the number of animals examined and the relevance of the paradigm.

24   TCE-induced increases in liver weight have been reported to occur quickly. The
25   inhalation study of Okino et al. (1991) in male rats demonstrates that liver weight and metabolism
26   were increased with as little as 8 hours of TCE exposure (500 and 2000 ppm) and as early as 22
27   hours after cessation of such exposures with little concurrent hepatic necrosis. Laughter reported
28   increase liver weight in SV129 mice in their 3-days study (see below). Tao et al. (2000) reported
29   a 1.26-fold of control % liver/body weight in female B6C3F1mice fed 1000 mg/kg TCE in corn
30   oil for 5 days. Elcombe et al. (1985) and Dees and Travis (1993) reported gavage results in mice
31   and rats after 10 days exposure to TCE which showed TCE-induced increases in liver weight (see
32   below for more detail on dose-response). Tucker et al (1982) reported that 14 days of exposure to
33   24 mg/kg and 240 mg/kg TCE via gavage to induce a dose-related increase in liver weight in male
34   CD-1 mice but did not show the data.

36   TCE-induced increases in % liver/body weight ratios have been studied most extensively
37   in B6C3F1 and Swiss mice. Both strains have been shown to have a TCE-induced increase in
38   liver tumors from long-term exposure as well (see Section 2.4.2. below). A number of studies
39   have provided dose-response information for TCE-induced increases in liver weight from 10 days
40   to 13 weeks of exposure in mice. Most studies have reported that the magnitude of increase in
41   TCE exposure concentration is similar to the magnitude increase of % liver/body weight increase.
42   For example a 2-fold increase in TCE exposure has often resulted in a 2-fold increase in the %
43   change in liver/body weight over control (i.e., 500 mg/kg TCE induces a 20% increase in liver

1   weight and 1000 mg/kg TCE induces a 50% increase in liver weight as reported by Elcombe et
2   al., 1985). The range in which this relationship is valid has been reported to vary from 100 mg/kg
3   TCE at 10 days (Dees and Travis, 1993) to 1600 mg/kg (Buben and O'Flaherty, 1985) at 6 weeks
4   and up to 1500 mg/kg TCE for 13 weeks (NTP, 1990). The consistency in the relationship
5   between magnitude of liver weight increase and TCE exposure concentration has been reported
6   for both genders of mice, across oral and inhalation routes of exposure, and across differing
7   strains of mice tested. For rats, there are fewer studies with fewer exposure levels tested, but both
8   Berman et al. (1995) and Melnick et al. (1987) report that short-term TCE exposures from 150
9   mg/kg to ~ 2000 mg/kg induced % liver/body weight that increased proportionally with the
10   magnitude of TCE exposure concentration.
11
12   Dependence of PPARα activation for TCE-liver weight gain has been investigated in
13   PPARα null mice by both Nakajima et al. (2000) and Laughter et al (2004). After 2 weeks of 750
14   mg/kg TCE exposure to carefully matched SV129 wild type or PPARα-null male and female mice
15   (n= 6 group), there was a reported 1.50-fold of control in wild-type and 1.26-fold of control %
16   liver/body weight in PPARα-null male mice by Nakajima et al. (2000). For female mice, there
17   was ~ 1.25-fold of control % liver/body weight ratios for both wild-type and PPARα-null mice.
18   Thus, TCE-induced liver weight gain was not dependent on a functional PPARα receptor in
19   female mice and some portion of it may have been in male mice. Both wild-type male and female
20   mice were reported to have similar increases in the number of peroxisome in the pericentral area
21   of the liver and TCE exposure and, although increased 2-fold, were still only ~ 4% of cytoplasmic
22   volume. Female wild type mice were reported to have less TCE-induced elevation of very long
23   chain acyl-CoA synthetase, D-type peroxisomal bifunctional protein, mitochondrial trifunctional
24   protein α subunits α and β, and cytochrome P450 4A1 than males mice, even though peroxisomal
25   volume was similarly elevated in male and female mice. The induction of PPARα protein by
26   TCE treatment was also reported to be slightly less in female than male wild-type mice (2.17-fold
27   vs. 1.44-fold of control, respectively).
28
29   Laughter et al (2004) also studied SV129 wild type and PPARα-null male mice treated
30   with 3 daily doses of TCE in 0.1% methyl cellulose for either 3 days (1500 mg/kg TCE) or 3
31   weeks (0, 10, 50, 125, 500, 1000, or 1500 mg/kg TCE 5 days a week). However, not only is the
32   paradigm not comparable to other gavage paradigms, but no initial or final body weights of the
33   mice were reported and thus the influence of differences in initial body weight on % liver/body
34   weight determinations could not be ascertained. In the 3-day study, while control wild type and
35   PPARα-null mice were reported to have similar % liver/body weight ratios (~ 4.5%), at the end of
36   the 3-week experiment the % liver/body weight ratios were reported to be increased in the
37   PPARα-null male mice (5.1%). TCE treatment for 3 days was reported to increase the %
38   liver/body weight ratio 1.4-fold of control in the wild type mice and 1.07-fold of control in the
39   null mice. In the 3-week study, wild-type mice exposed to various concentrations of TCE had %
40   liver/body weights that were reported to be within ~ 2% of control values except for the 1000
41   mg/kg and 1500 mg/kg groups (~ 1.18 – fold and 1.30 - fold of control levels, respectively). For
42   the PPARα-null mice the variability in % liver/body weight was reported to be greater than that of
43   the wild-type mice in most of the groups and the baseline level of % liver/body weight ratio also

1   1.16-fold greater. TCE exposure was apparently more toxic in the null mice with death at the
2   1500 mg/kg TCE exposure level resulting in the prevention of recording of % liver/body weights.
3   At 1000 mg/kg TCE exposure level there was a reported 1.10-fold of control % liver/body weight
4   in the PPARα-null mice. None of the increases in % liver/body weight in the null mice were
5   reported to be statistically significant by Laughter et al. (2004). However, the statistical power of
6   the study was limited due to low numbers of animals and increased variability in the null mice
7   groups. The % liver/body weight after TCE treatment that was reported in this study was actually
8   greater in the null mice than the wild-type male mice at the 1000 mg/kg TCE exposure level (5.6
9    $\pm$ 0.4 % vs. 5.2 $\pm$ 0.5%, for null and wild-type mice, respectively). At 1-weeks and at 3-weeks,
10   TCE appeared to induce increases in liver weight in PPARα-null mice, although not reaching
11   statistical significance in this study. At a 1000 mg/kg TCE exposure for 3 weeks % liver/body
12   weights were reported to be 1.18-fold of control in wild-type and 1.10-fold of control in null
13   mice. Although the experiments in Laughter et al. for DCA and TCA were not conducted using
14   the same paradigm, the TCE-induced increase in % liver/body weight more closely resembled the
15   dose-response pattern for DCA than for DCA wild-type SV129 and PPARα-null mice.
16
17   Many studies have used cyanide-insensitive palmitoyl CoA oxidase activity (PCO) as a
18   surrogate for peroxisome proliferation. Of note is that several studies have shown that this
19   activity is not correlated with the volume or number of peroxisomes that are increased as a result
20   of exposure to TCE or it metabolites (Nakajima et al 2000; Elcombe et al 1985: Nelson et al
21   1989).   This activity appears to be highly variable both as a baseline measure and in response to
22   chemical exposures. Laughter et al. (2004) presented data showing that WY-14,643 induced
23   increases in PCO activity varied up to 6-fold between experiments in wild-type mice. They also
24   showed that PCO activity, in some instances, was up to 6-fold of wild type mice values in
25   untreated PPARα-null mice. Parrish et al. (1996) noted that control values between experiments
26   varied as much as a factor of 2-fold for PCO activity and thus their data were presented as percent
27   of concurrent controls. Goldsworthy and Popp (1987) reported that 1000 mg/kg TCE induced a
28   6.25-fold of control PCO activity in B6C3F1 mice in two 10-day experiments. However for F344
29   rats, the increases over control between two experiments conducted at the same dose were
30   reported to vary by > 30%. Finally, Melnick et al. (1987) have reported that corn oil
31   administration alone can elevate PCO activity as well as catalase activity.
32
33   For TCE there are two key 10-days studies (Elcombe et al., 1985; Dees and Travis, 1993)
34   that examine the effects of short-term exposure in mice and rats via gavage exposure and attempt
35   to determine the nature of the dose response in a range of exposure concentrations that include
36   levels below which there is concurrent decreased body weights. Although they have limitations,
37   they reported generally consistent results. In regard to liver weight in mice, gavage exposure to
38   TCE at concentrations ranging from 100 to 1500 mg/kg TCE produced increases in liver/body
39   weight that was dose-related (Elcombe et al., 1985; Dees and Travis, 1993).
40
41   Elcombe et al. (1985) reported a small decrease in DNA content with TCE treatment
42   (consistent with hepatocellular hypertrophy) that was not dose-related, increased tritiated
43   thymidine incorporation in whole mouse liver DNA that was that was treatment but not dose-

1   related (i.e., a 2-, 2-, and 5-fold of control values in mice treated with 500, 1000, and 1500 mg/kg
2   TCE), and slightly increased numbers of mitotic figures that were treatment but not dose-related
3   and not correlated with DNA synthesis as measured by thymidine incorporation. Elcombe et al
4   (1985) reported an increase in peroxisome volume after TCE exposure that was correlated with
5   the magnitude of increase in peroxisomal-associated enzyme activity at the only dose in which
6   both were tested. Peroxisome increases after TCE treatment in mice livers were identified as
7   being pericentral in location. After TCE treatment, increased peroxisomal volumes in B6C3F1
8   mice were reported to be not dose-related (i.e., there was little difference between 500 to 1500
9   mg/kg TCE exposures). The TCE-induced increases in peroxisomal volumes were also not
10 correlated with the reported increases in thymidine incorporation or mitotic activity in mice.
11 Neither TCE-induction of peroxisomes or hepatocellular proliferation, as measured by either
12 mitotic index or thymidine incorporation, was correlated with TCE-induced liver weight
13 increases. Elcombe et al. (1985) only measured PCO activity in a subset of B6C3F1 mice at the
14 1000 mg/kg TCE exposure level for 10 days of exposure and reported an 8-fold of control PCO
15 activity and a 1.5-fold of control catalase activity. This result was similar to that of Goldsworthy
16 and Popp (1987) who reported 6.25-fold of control PCO activity in male B6C3F1 mice exposed
17 to 1000 mg/kg/day TCE for 10 days in two separate experiments.
18
19   Similar to Elcombe et al., who reported no difference in response between 500 and 1000
20 mg/kg TCE treatments, Dees and Travis (1993) reported that incorporation of tritiated thymidine
21 in DNA from mouse liver was elevated after TCE treatment and the mean peak level of tritiated
22 thymidine incorporation occurred at 250 mg/kg TCE treatment level remaining constant for the
23 500 and 1000 mg/kg treated groups. Dees and Travis (1993) specifically report that mitotic
24 figures, although very rare, were more frequently observed after TCE treatment, found most often
25 in the intermediate zone, and found in cells resembling mature hepatocytes. They reported that
26 there was little tritiated thymidine incorporation in areas near the bile duct epithelia or close to the
27 portal triad in liver sections from both male and female mice. They also reported no evidence of
28 increased lipofuscin and that increased apoptoses from TCE exposure "did not appear to be in
29 proportion to the applied TCE dose given to male or female mice" (i.e., the mean number of
30 apopotosis 0, 0, 0, 1 and 8 for control, 100, 250, 500, and 1000 mg/kg TCE treated groups,
31 respectively). Both Elcombe et al. (1985) and Dees and Travis (1993) reported no changes in
32 apoptosis other than increased apoptosis only at a treatment level of 1000 mg/kg TCE.
33
34   Elcombe et al. (1985) reported increased in % liver/body weight after TCE treatment in
35 both the Osborne-Mendel and Alderly Park rat strain, although to a smaller extent than in mice.
36 For both strains, Elcombe et al. (1985) reported no TCE-induced changes in body weight at doses
37 ranging from 500 to 1500 mg/kg. For male Osborne-Mendel rats administration of TCE in corn
38 oil gavage resulted in a 1.18-fold, 1.26-fold, and 1.30-fold of control % liver/body weight at 500
39 mg/kd/day, 1000 mg/kg/day, and 1500 mg/kg/day exposures, respectively. For Alderly Park rats
40 those increases were 1.14-fold, 1.17-fold, and 1.17-fold of control at the same respective exposure
41 levels for 10 days of exposure. In regard to liver weight increases, Melnick et al (1987) reported a
42 1.13-fold and 1.23-fold of control % liver/body weight in male Fischer 344 rats fed 600
43 mg/kg/day and 1300 mg/kg/day TCE in capsules, respectively. There was no difference in the

1   extent of TCE-induced liver increase between the two lowest dosed group administered TCE in
2   corn oil gavage (~ 20% increase in % liver/body weight at 600 mg/kd and 1300 mg/kg TCE) for
3   14 days. However, the magnitude of increases in % liver/body weight in these groups was
4   affected by difference between control groups in liver weight although initial and final body
5   weights appeared to be similar. By either type of vehicle, Melnick et al. (1987) reported
6   decreases in body weights in rats treated with concentrations of TCE 2200 mg/kg/day or greater
7   for 14 days. Similarly, Nunes et al (2001) reported decreased body weight in Sprague-Dawley
8   rats administered 2000 mg/kg/day for 7 days in corn oil. Melnick et al (1987) reported that both
9   exposures to either 600 or 1300 mg/kg/day TCE in capsules did not result in decreased body
10 weight and caused less than minimal focal necrosis randomly distributed in the liver. At 2200 and
11 4800 mg/kg TCE fed via capsule, Melnick et al. (1987) reported that although there was
12 decreased body weight in rats treated at these exposures, there was little TCE-induced necrosis,
13 and no evidence of inflammation, cellular hypertrophy or edema with TCE exposure. Similarly,
14 Berman et al. (1995) reported increases in liver weight gain at doses as low as 50 mg/kg TCE, no
15 necrosis up to doses of 1500 mg/kg, and hepatocellular hyper trophy only at the 1500 mg/kg level
16 in female Fischer 344 rats.
17
18   For rats, Elcombe et al. (1985) reported an increase over untreated rats of 1.13-fold of
19 control PCO activity in Alderly Park rats after 1000 mg/kg/day TCE exposure for 10 days, while
20 Goldsworthy and Popp (1987) reported a 1.8-fold and 2.39-fold of control in male Fischer 344
21 rats at the same exposure in two separate experiments. Melnick et al. (1987) reported PCO
22 activity of 1.23-fold and 1.75-fold of control in male Fischer 344 rats fed 600 mg/kg/day and
23 1300 mg/kg/day TCE for 14 days in capsules. For rats treated by gavage with 600 mg/kg/day or
24 1200 mg/kg day TCE corn oil, they reported 1.16-fold and 1.29-fold of control values. However,
25 control levels of PCO were 16% higher in corn oil controls than in untreated controls. In addition
26 Melnick et al. (1987) reported little catalase increases in rats fed TCE via capsules in food (less
27 than 6% increase) but a 1.18-fold and 1.49-fold of control catalase activity in rats fed 600
28 mg/kg/day or 1200 mg/kg/TCE via corn oil gavage, indicative of a vehicle effect.
29
30   The data from Elcombe et al.(1985) included reports of TCE-induced pericentral
31 hypertrophy and eosinophilia for both rats and mice but with "fewer animals affected at lower
32 doses." In terms of glycogen deposition, Elcombe report "somewhat" less glycogen pericentrally
33 in the livers of rats treated with TCE at 1500 mg/kg than controls with less marked changes at
34 lower doses restricted to fewer animals. They do not comment on changes in glycogen in mice.
35 Dees and Travis (1993) reported TCE-induced changes to "include an increase in eosinophilic
36 cytoplasmic staining of hepatocytes located near central veins, accompanied by loss of
37 cytoplasmic vacuolization." Since glycogen is removed using conventional tissue processing and
38 staining techniques, an increase in glycogen deposition would be expected to increase
39 vacuolization and thus the report from Dees and Travis is consistent with less not more glycogen
40 deposition. Neither study produced a quantitative analysis of glycogen deposition changes from
41 TCE exposure.   Although not explicitly discussing liver glycogen content or examining it
42 quantitatively in mice, these studies suggest that TCE-induced liver weight increases did not

1   appear to be due to glycogen deposition after 10 days of exposure and any decreases in glycogen
2   were not necessarily correlated with the magnitude of liver weight gain either.
3
4   For both rats and mice the data from Elcombe et al (1985) showed that tritiated thymidine
5   incorporation in total liver DNA observed after TCE exposure did not correlate with mitotic index
6   activity in hepatocytes with both Elcombe et al. (1985) and Dees and Travis (1993) reporting a
7   small mitotic indexes and evidence of periportal hepatocellular hypertrophy from TCE exposure.
8   Neither mitotic index or tritiated thymidine incorporation data support a correlation with TCE-
9   induced liver weight increase in the mouse. If higher levels of hepatocyte replication had
10   occurred earlier, such levels were not sustained by 10 days of TCE exposure. Both Elcombe et al.
11   (1985) and Dees and Travis (1993) present data that represent "a snapshot in time" which does
12   not show whether increased cell proliferation may have happened at an earlier time point and then
13   subsided by 10 days. These data suggest that increased tritiated thymidine levels were targeted to
14   mature hepatocytes and in areas of the liver where greater levels of polyploidization occur. Both
15   Elcombe et al.(1985) and Dees and Travis (1993) show that tritiated thymidine incorporation in
16   the liver was ~ 2-fold of controls between 250 -1000 mg/kg TCE, a result consistent with a
17   doubling of DNA. Thus, given the normally quiescent state of the liver, the magnitude of this
18   increase over control levels, even if a result of proliferation rather than polyploidization, would be
19   confined to a very small population of cells in the liver after 10 days of TCE exposure. Laughter
20   et al. (2004) reported that there was an increase in DNA synthesis after aqueous gavage exposure
21   to 500 and 1000 mg/kg TCE given as 3 boluses a day for 3 weeks with BrdU given for the last
22   week of treatment. An examination of DNA synthesis in individual hepatocytes was reported to
23   show that 1% and 4.5% of hepatocytes had undergone DNA synthesis in the last week of
24   treatment for the 500 and 1000 mg/kg doses, respectively. Both Elcombe et al (1985) and Dees
25   and Travis (1993) show TCE-induced changes for several parameters at the lowest level tested
26   without toxicity and without evidence of regenerative hyperplasia or sustained hepatocellular
27   proliferation. In regards to susceptibility to liver cancer induction, the more susceptible
28   (B6C3F1) vs. less susceptible (Alderly Park/Swiss) strains of mice to TCE-induced liver tumors
29   (Maltoni et al., 1988), the "less susceptible" strain was reported by Elcombe et al (1985) to have,
30   a greater baseline level of liver weight/body weight ratio, a greater baseline level of thymidine
31   incorporation as well as greater responses for those endpoints due to TCE exposure. However,
32   both strains showed a hepatocarcinogenic response after TCE exposure, although there are
33   limitations regarding determination of the exact magnitude of response for these experiments as
34   previously discussed.
35
36   **2.4.2. Summary of Results For Short-term Effects of DCA and TCA: Comparisons With**
37   **   TCE**
38
39   Short-term exposures from DCA and TCA have been studied either through gavage or in
40   drinking water. Palatability became an issue at the highest level of DCA tested in drinking water
41   experiments (5 g/l) which caused a significant reduction of drinking water intake in mice of 46 to
42   64% (Carter et al 1995). Decreases in drinking water consumption have also been reported for a
43   range of concentrations of DCA and TCA from 0.05 g/l to 5.0 g/l, in both mice and rats, and with

1 generally the higher concentrations producing the highest decrease in drinking water (Carter et al.,
2 1995; Mather et al., 1990; DeAngelo et al., 1997; DeAngelo et al., 1999; DeAngelo et al., 2008).
3 However, results within studies (e.g., DeAngelo et al., 2008) and between studies have been
4 reported to vary as to the extent of the reduction in drinking water from the presence of TCA or
5 DCA. Some drinking water studies of DCA or TCA have not reported drinking water
6 consumption as well. Therefore, although in general DCA and TCA studies have do not include
7 vehicle effects, such as corn oil, they have been affected by differences in drinking water
8 consumption not only changing the dose received by the rodents and therefore potentially the
9 shape of the dose-response curve, but also the effects of dehydration are potentially added to any
10 chemically-related reported effects.
11
12   Studies have attempted to determine short-term effects on DNA by TCE and its
13 metabolites. Nelson and Bull (1988) administered TCE male Sprague Dawley rats and male
14 B6C3F1 mice measured the rate of DNA unwinding under alkaline conditions 4 hours later. For
15 rats there was a significantly increased rate of unwinding at the two highest dose and for mice
16 there was a significantly increased level of DNA unwinding at a lower dose. In this same study,
17 DCA was reported to be most potent in this assay with TCA being the lowest, while chloral
18 hydrate (CH) closely approximated the dose-response curve of TCE in the rat. In the mouse the
19 most potent metabolite in the assay was reported to be TCA followed by DCA with CH
20 considerably less potent. Nelson and Bull (1988) and Nelson et al (1989) have reported increases
21 in single strand breaks after DCA and TCA exposure. However, Styles et al (1991) (for mice)
22 and Chang et al (1992) (for mice and rats) did not. Austin et al. (1996) note that the alkaline
23 unwinding assay, a variant of the alkaline elution procedure, is noted for its variability and
24 inconsistency depending on the techniques used while performing the procedure. In regard to
25 oxidative damage as measured by TBARS for lipid peroxidation and 8-OHdG levels in DNA,
26 increases appear to be small (less than 50% greater than control levels) and transient after DCA
27 and TCA treatment in mice (see Section 3.4.2.3) with TCE results confounded by vehicle or route
28 of administration effects.
29
30   Although there is no comparative data for TCE, the study of Styles et al. (1991) is
31 particularly useful for determining effects of TCA from 1 to 4 days of exposure in mice. Styles et
32 al (1991) reported no change in "hepatic" DNA uptake of tritiated thymidine up to 36 hours, a
33 peak at 72 hours (~ 6-fold of control), and falling levels by 96 hours (~ 4-fold of controls) after
34 500 mg/kg TCA gavage exposure. Incorporation of tritiated thymidine observed for individual
35 hepatocytes decreased between 24 and 36 hours, rose slowly back to control levels at 48 hours,
36 significantly increased by 72 hours, and then decreased by 96 hours. Thus, increases in "hepatic"
37 DNA tritiated thymidine uptake did not capture the decrease observed in individual hepatocytes at
38 36 hours. By either measure the population of cells undergoing DNA synthesis was small with
39 the peak level being less than 1% of the hepatocyte population. Zonal distribution of labeled
40 hepatocytes were decreased at 36 hours in all zones, appeared to be slightly greater in perioportal
41 than midzonal cells with centrilobular cells still below control levels by 48 hours, similarly
42 elevated over controls in all zones by 72 hours, and to have returned to near control levels in the
43 midzonal and centrilobular regions but with periportal areas still elevated by 96 hours. These

1 results are consistent with all hepatocytes showing a decrease in DNA synthesis by 36 hours and
2 then a wave of DNA synthesis to occur, starting at the periportal zone and progressing through the
3 liver acinus that is decreased by 4 days after exposure.
4
5 　　Along with changes in liver weight, DNA synthesis, and glycogen accumulation, several
6 studies of DCA and TCA have focused on the extent of peroxisome proliferation as measured by
7 changes in peroxisome number, cytoplasmic volume and enzyme activity induction as potential
8 "key events" occurring from shorter-term exposures that may be linked to chronic effects such as
9 liver tumorigenicity. As noted above in Section 2.4.1, TCE-induced liver weight gain has been
10 reported to not be dependent on a functional PPARα receptor in female mice while some portion
11 of increased liver weight may have been in male mice. Also as noted cyanide-insensitive
12 palmitoyl CoA oxidase activity (PCO) has also been reported to not be correlated with the volume
13 or number of peroxisomes that are increased as a result of exposure to TCE or it metabolites
14 (Nakajima et al 2000; Elcombe et al 1985: Nelson et al 1989) and to be highly variable both as a
15 baseline measure and in response to chemical exposures (e.g., variation of up to 6-fold between
16 after WY-14,643 exposure in mice). Also as noted, above the vehicle used in many TCE gavage
17 experiments, corn oil, has been reported to elevate PCO activity as well as catalase activity.
18
19 　　A number of short-term studies have examined the effects of TCA and DCA on liver
20 weight increases and evidence of peroxisome proliferation and changes in DNA synthesis. In
21 particular two studies of DCA and TCA used a similar paradigm presented by Elcombe et al
22 (1985) and Dees and Travis (1993) for TCE effects in mice. Nelson et al. (1989) report findings
23 from gavage doses of unbuffered TCA (500 mg/kg) and DCA (500 mg/kg) in male B6C3F1 mice
24 and Styles et al. (1991) also providing data on peroxisome proliferation using the same paradigm.
25 Nelson et al. (1989) reported levels of PCO activity in mice administered 500 mg/kg DCA or
26 TCA for 10 days with 250 mg/kg Clofibrate administration serving as a positive control. DCA
27 and TCA exposure were reported to not affect body weight, but both to significantly increase liver
28 weight (1.63-fold of control for DCA and 1.30-fold of control for TCA treatments), and %
29 liver/body weight ratios (1.53-fold of control for DCA and 1.16-fold of control for DCA
30 treatments). PCO activity was reported to be significantly increased by ~ 1.63-fold, 2.7-fold, and
31 5-fold of control for DCA, TCA and Clofibrate treatments, respectively and indicated that both
32 DCA and TCA were weaker inducers of this activity than Clofibrate. Results from randomly
33 selected electron photomicrographs showed an increase in peroxisomes per unit area but gave a
34 different pattern than PCO enzyme activity (i.e., 2.5-fold and 2.4-fold of control peroxisome
35 volume for DCA and TCA, respectively). Evidence of gross hepatotoxicity was reported to not
36 occur in vehicle or TCA-treated mice. Light microscopic sections were reported to show TCA
37 and control hepatocytes to have the same intensity of PAS staining, but with slightly larger
38 hepatocytes occurring in TCA-treated mice throughout the liver section with architecture and
39 tissue pattern of the liver intact. For DCA, the histopathology was reported to be markedly
40 different than control mice or TCA treated mice. DCA was reported to induce a marked increase
41 in the size of hepatocytes throughout the liver with an approximately 1.4-fold of control diameter
42 that was accompanied by increased PAS staining (indicative of glycogen deposition). All DCA-
43 treated mice were reported to have multiple white streaks grossly visible on the surface of the

1 liver corresponding with subcapsular foci of coagulative necrosis that were not encapsulated,
2 varied in size, and accompanied by a slight inflammatory response characterized by neutrophil
3 infiltration.
4
5   A quantitative comparison of effects from equivalent exposures of TCE, TCA, and DCA
6 (500 mg/kg for 10 days in mice via corn oil gavage for TCE) can be drawn between the Elcombe
7 et al. (1985), Dees and Travis (1993), Styles et al. (1991), and Nelson et al. (1989) data for
8 relationship to control values for % liver/body weight, peroxisome volume and activity (PCO),
9 and qualitatively for glycogen deposition.
10

Model		% Liver/ Body Wt.	Peroxisome Volume	Peroxisome Enzyme Activity	Glycogen Deposition
Nelson et al. (1989)**					
B6C3F1 Male	TCA	1.16-fold	2.4-fold	2.7-fold	no change
	DCA	1.53 -fold	2.5-fold	1.63-fold	increased
Styles et al. (1991)					
B6C3F1 Male	TCA	NR	1.9-fold	NR	NR
Elcombe et al. (1985)					
B6C3F1 Male	TCE	1.20-fold	8-fold	NR	NR
Alderly Park Male (Swiss)	TCE	1.43-fold	4-fold	NR	NR
Dees and Travis (1993)					
B6C3F1 Male	TCE	1.05-fold*	NR	NR	NR
B6C3F1 Female	TCE	1.18-fold	NR	NR	NR

1   * statistically significant although small increase. ** Unbuffered. NR = not reported as no
2   analysis was performed for this dose or the authors did not report this finding (i.e., did not note a
3   change in glycogen in description of exposure-related changes).
4
5   Although using a similar species, route of exposure, and dose, the comparison of
6   responses for TCE and it's metabolites shown above are in male mice and also are reflective of
7   variability in strain, and variability and uncertainty of initial body weights. As described in more
8   detail in Section 2.2, initial age and body weight have an impact on TCE-related increases in liver
9   weight. Male mice have been reported to have greater variability in response than female mice
10 within and between studies and most of the comparative data for the 10-day 500 mg/kg doses of
11 TCE or its metabolites were from studies in male mice. Corn oil, used as the vehicle for TCE
12 gavage studies but not those of its metabolites, has been noted to specifically affect peroxisomal
13 enzyme induction, body weight gain, and hepatic necrosis, specifically, in male mice (Merrick et
14 al 1989). Corn oil alone has also been reported to increase PCO activity in F344 rats and to
15 potentiate the induction of PCO activity of TCA (DeAngelo et al., 1989). Thus, quantitative
16 inferences regarding the magnitude of response in these studies are limited by a number of
17 factors.
18
19   The variability in the magnitude of TCE-induced increases in % liver/body weight across
20 studies in readily apparent but for TCE, TCA and DCA there is an increase in liver weight in mice
21 at this dose after 10 days of exposure. The volume of the peroxisomal compartment in
22 hepatocytes was reported to be more greatly increased from TCE-treatment by Elcombe et al.
23 (1985) than for either TCA or DCA by Nelson et al. (1989) or Styles et al. (1991). However, the
24 control values for the B6C3F1 mice were half that of the other strain reported by Elcombe et al.
25 (1985) and this parameter in general did not match the pattern of PCO activity values reported for
26 TCA and DCA (Nelson et al., 1989). There is no PCO activity data at this dose for TCE but
27 Elcombe et al. (1985) reported that the magnitude of TCE-induced increase in peroxisome
28 volume was similar to that of PCO activity at the only dose where both were tested (1000 mg/kg
29 TCE). However, Elcombe et al. (1985) reported increased peroxisomal volumes in B6C3F1 mice
30 after 10 days of TCE treatment were not dose-related (i.e., there was little difference between 500,
31 1000 and 1500 mg/kg TCE exposures in the magnitude of TCE-induced increases in peroxisomal
32 volume). The lack of dose-response for TCE-induced peroxisomal volume increases was not
33 consistent with increases in % liver/body weight that increased with increasing TCE exposure
34 concentration. Also as noted above, PCO activity appears to be highly variable in untreated and
35 treated rodents and to vary between experiments and between studies.
36
37   From the above comparison it is clears that TCE, DCA and TCA exposures were
38 associated with increased liver weight in mice but a question arises as to what changes account
39 for the liver weight increases. For TCE and TCA 500 mg/kg treatments, changes in glycogen
40 were not reported in the general descriptions of histopathological changes (Elcombe et al., 1985;
41 Styles et al., 1991; Dees and Travis, 1993) or were specifically described by the authors as being
42 similar to controls (Nelson et al., 1989). However for DCA, glycogen deposition was specifically
43 noted to be increased with treatment, although no quantitative analyses was presented that could

1   give information as to the nature of the dose-response (Nelson et al., 1989). Issues in regard to
2   not only whether TCE and its metabolites each gives a similar response for a number of
3   parameters, but what potential changes may be associated with carcinogenicity from long-term
4   exposures can be examined by a comparison of the dose-response curves for these parameters
5   from a range of exposure concentrations and durations of exposure. In addition, if glycogen
6   accumulation results from DCA exposure, what proportion of DCA-induced liver weight
7   increases result from such accumulation or other events that may be similar to those occurring
8   with TCE exposure (see Section 4.2.4 below)?
9

10   As noted above in Section 2.4.1., TCE-induced changes in liver weight appear to be
11   proportional to the exposure concentration across route of administration, gender and rodent
12   species. As an indication of the potential contribution of TCE metabolites to this effect, a
13   comparison of the shape of the dose-response curves for liver weight induction for TCE and its
14   metabolites is informative. A number of studies of TCA and DCA in drinking water, conducted
15   from 10-days to 4 weeks, have attempted to measure changes in liver weight induction,
16   peroxisomal enzyme activity, and changes in DNA synthesis predominantly in mice to provide
17   insight into the MOA(s) for liver cancer induction (Parrish et al., 1996; Sanchez and Bull 1990;
18   Carter et al 1995; DeAngelo et al.,1989, 2008).
19

20   Direct comparisons are harder to make between the gavage studies of TCE for 10 days of
21   exposure and those of DCA and TCA via drinking water. Similar to 10-day gavage exposures to
22   TCE, 14-day exposures to TCA or DCA via drinking water were reported to induce dose-related
23   increases in liver weight in male B6C3F1 mice (0.3, 1.0 , and 2.0 g/l TCA or DCA) with a greater
24   increase in liver weight from DCA than TCA at 2 g/l and a difference in the shape of the dose-
25   response curve (Sanchez and Bull, 1990). They reported a 1.08-fold, 1.31-fold, and 1.62-fold of
26   control liver weight for DCA and a 1.15-fold, 1.22-fold, and 1.38-fold of control values for TCA
27   at 0.3 g/l, 1.0 g/l and 2.0 g/l concentrations, respectively (n= 12-14 mice). While the magnitude
28   of difference between the exposures was ~ 6.7-fold between the lowest and highest dose, the
29   differences between TCA exposure groups for change in % of liver weight was ~ 2.5, but for
30   DCA the slope of the dose-response curve for liver weight increases appeared to be closer to the
31   magnitude of difference in exposure concentrations between the groups (i.e. a difference of 7.7-
32   fold between the highest and lowest dose for liver weight induction).
33

34   DeAngelo et al. (1989) reported that after 14 days of exposure to 5 g/l or 2 g/l TCA in
35   male mice, the magnitudes of the difference in the increase in dose (2.5-fold) was generally
36   higher than the increase % liver/body weight ratios at these doses (i.e., ~ 40% for the Swiss-
37   Webster, C3H, and for one of the B6C3F1 mouse experiments, and for the C57BL/6 mouse there
38   was no difference in liver weight induction between the 2 g/l and 5g/l TCA exposure groups).
39   There was a range in the magnitude of % liver/ body weight ratio increases between the strains of
40   mice with liver weight induction reported to range between 1.26-fold to 1.66-fold of control
41   values for the 4 strains of mice at 5 g/l TCA and to range between 1.16-fold to 1.63-fold of
42   control values at 2 g/l TCA. One strain, B6C3F1, was chosen to compare responses between
43   DCA and TCA. At 1 g/l, 2 g/l and 5 g/l TCA or DCA, DCA was reported to induce a greater

1   increase in liver weight that TCA (i.e. 1.55-fold vs. 1.39-fold of control % liver/body weight ratio
2   for 5.0 g/l DCA vs. TCA, respectively). At the 5 g/l exposures DCA induced ~ 40% greater %
3   liver/body weight than TCA. Although as noted above, the majority of the data from this study in
4   mice did not indicate that the magnitude of difference in exposure concentration was the same as
5   that of liver weight induction for TCA, in the particular experiment that examined both DCA and
6   TCA, the increase in % liver/body weight ratios were similar to the magnitude of difference in
7   dose between the 2 g/l and 5 g/l exposure concentrations for both DCA and TCA (i.e. 2- to 2.5-
8   fold increase in liver weight change corresponding to a 2.5-fold difference in exposure
9   concentration).
10
11   Carter et al. (1995) examined 0.5 g/l and 5.0 g/l exposures to DCA in B6C3F1 male mice
12 and reported that % liver/body weights were increased consistently from 0.5 g/l DCA treatment
13 from 5 days to 30 days of treatment (i.e., a range of 1.05-fold to 1.16-fold of control). For 5.0 g/l
14 DCA exposure the range of increase in % liver/body weight was reported to be 1.37-fold to 2.04-
15 fold of control for the same time period. At the 15 days of exposures the % liver/body weight
16 ratios were 1.67-fold and 1.12-fold of control for 5.0 g/l and 0.5 g/l DCA and at 30 days were
17 1.99-fold and 1.16-fold, respectively.   The difference in magnitude of dose and % liver/body
18 weight increase is difficult to determine given that the 5 g/l dose of DCA reduced body weight
19 and significantly reduced water consumption by ~ 50%. The differences in DCA-induced %
20 liver/body weights were ~ 6-fold for the 15, 25, and 30-day data between the 0.5 and 5 g/l DCA
21 exposures rather than the 10-fold difference in exposure concentration in the drinking water.
22
23   Parrish et al (1996) reported that for male B6C3F1 mice exposed to TCA or DCA (0, 0.01,
24 0.5 and 2.0 g/l) for 3 or 10 weeks, the 4- to 5-fold magnitude of difference in doses resulted in
25 increases in % liver/body weight for the 21-day and 71-day exposures that were greater for DCA
26 than TCA. The % liver/body weight ratio were 0.98-fold, 1.13-fold, 1.33-fold of control levels at
27 0.1 g/l, 0.5 g/l and 2.0 g/l TCA and for DCA were 1.02-fold, 1.24-fold, and 1.46-fold of control
28 levels, respectively, after 21 days of exposure. Both TCA and DCA exposures at 0.1 g/l resulted
29 in difference in % liver/body weight change of 2% or less. For TCA, although there was a 4-fold
30 increase in magnitude between the 0.5 and 2.0 g/l TCA exposure concentrations, the magnitude of
31 increase for % liver/body weight increase was 2.5-fold between them at both 21 and 71 days of
32 exposure. For DCA, the 4-fold difference in dose between the 0.5 and 2.0 g/l DCA exposure
33 concentrations were reported to result in a ~ 2-fold increase in % liver/body weight increase at 21
34 days and ~ 4.5-fold increase at 71 days.
35
36   DeAngelo et al. (2008) studied 3 exposure concentrations of TCA in male B6C3F1 mice,
37 which were an order of magnitude apart, for 4 weeks of exposure.   The % liver/body weight
38 ratios were 1.09-fold, 1.16-fold, and 1.35-fold of control levels, for 0.05, 0.5, and 5.0 g/l TCA
39 exposures, respectively. The 10-fold differences in exposure concentration of TCA resulted in ~
40 2-fold differences in % liver/body weight increases. No dose-response inferences can be drawn
41 from the 4-week study of DCA and TCA in B6C3F1 male mice by Kato-Weinstein et al. (2001)
42 but 2 g/l DCA and 3 g/l TCA in drinking water were reported to induce % liver/body weights of
43 1.42-fold and 1.33-fold of control, respectively (n=5).

1   The majority of short-term studies of DCA and TCA in mice have been conducted in the
2   B6C3F1 strain and in males. Studies conducted from 14 to 30 days show a consistent increase in
3   % liver/body weight induction by TCA or DCA. Accordingly an examination of all of the data
4   from Parrish et al. (1996), Sanchez and Bull (1990), Carter et al. (1995), Kato-Weinstein et al.
5   (2001), and DeAngelo et al. (1989; 2008) from 14 to 30 days of exposure in male B6C3F1 mice
6   can give an approximation of the dose-response differences between DCA and TCA for liver
7   weight induction as shown in the table below and figure. Although the data for B6C3F1 mice
8   from Sanchez and Bull (1990) is reported as the fold of liver weight rather that % liver/body
9   weight increase, it is included in the comparison as both reflect increase in liver weight. Similar
10   data can be assessed for TCE for comparative purposes. Short duration studies (10-42 days) were
11   selected because (i) in chronic studies, liver weight increases are confounded by tumor burden,
12   (ii) multiple studies are available, and (iii) in this duration range, Kjellstrand et al. (1981) reported
13   that TCE-induced increases in liver weight plateau, and (iv) TCA studies do not show significant
14   duration-dependent differences in this duration range. These comparisons are presented in the
15   table below.
16
17   DeAngelo et al (1989) and Carter et al (1995) used up to 5 g/l DCA and TCA in their
18   experiments with Carter et al (1995) noting a dramatic decrease in water consumption in the 5 g/l
19   DCA treatment groups (46%- 64% reduction) which can affect body weight as well as dose
20   received. DeAngelo et al (1989) did not report drinking water consumption. The drinking water
21   consumption was reported by DeAngelo et al. (2008) to be reduced by 11%, 17%, and 30% in the
22   0.05g/l, 0.5 g/l and 5 g/l TCA treated groups compared to 2 g/l NaCl control animals over 60
23   weeks. DeAngelo et al. (1999) reported mean drinking water consumption to be reduced by 26%
24   in mice exposed to 3.5 g/l DCA over 100 weeks. Carter et al. (1995) reported that DCA at 5 g/l to
25   decrease drinking water consumption by 64% and 46% but 0.5 g/l DCA to not affect drinking
26   water consumption. Thus it appears that the 5 g/l concentrations of either DCA or TCA can
27   significantly affect drinking water consumption as well as inducing reductions in body weight.
28   Accordingly, an estimation of the shape of the dose-response curve for comparative purposes
29   between DCA or TCA drinking water studies is best examined at concentrations at 2g/l or less,
30   especially for DCA.
31
32   The dose-response curves for similar concentrations of DCA and TCA are presented in
33   Figure 4.4.1 for durations of exposure from 14-28 days in the male B6C3F1 mouse, which was
34   the most common sex and strain used. For this comparative analysis an average is provided
35   between two values for a given concentration and duration of exposure for comparison with other
36   doses and time points. As noted in the discussion of individual experiments, there appears to be a
37   linear correlation between dose in drinking water and liver weight induction up to 2 g/l of DCA.
38   However, the shape of the dose-response curve for TCA appears to be quite different (i.e., lower
39   concentrations of TCA inducing larger increase that does DCA but then the response reaching an
40   apparent plateau for TCA at higher doses while that of DCA continues to increase). As shown by
41   DeAngelo et al (2008), 10-fold differences in the magnitude of exposure concentration to TCA
42   corresponded to ~ 2-fold differences in liver weight induction increases. In addition, TCA studies

1 did not show significant duration-dependent difference in liver weight induction in this duration
2 range as shown in the table below.
3
4   Of interest is the issue of how the dose-response curves for TCA and DCA compare to
5 that of TCE in a similar model and dose range. Since TCA and DCA have strikingly different
6 dose-response curves, which one if either best fits that of TCE and thus can give insight as to
7 which is causative agent for TCE's effects in the liver? In the case of the TCE database in the
8 mouse two strains have been predominantly studied, Swiss and B6C3F1, and both have been
9 reported to get liver tumors in response to chronic TCE exposure. Rather than administered in
10 drinking water, oral TCE studies have been conducted via oral gavage and generally in corn oil
11 for 5 days of exposure per week.   The study by Goel et al. (1992) was conducted in ground-nut
12 oil. Vehicle effects, the difference between daily and weekly exposures, the dependence of TCE
13 effects in the liver on its metabolism to a variety of agents capable inducing effects in the liver,
14 differences in response between strains, and the inherent increased variability in use of the male
15 mouse model all add to increased difficulty in establishing the dose-response relationship for TCE
16 across studies and for comparisons to the DCA and TCA database. Despite difference in
17 exposure route, etc, a consistent pattern of dose-response emerges from combining the available
18 TCE data. The effects of oral exposure to TCE from 10-42 days on liver weight induction is
19 shown in Figure 4.4.2 using the data of Elcombe et al (1985), Dees and Travis (1993), Goel et al.
20 (1992), Merrick et al. (1987), Goldsworthy and Popp (1987), and Buben and O'Flaherty (1985).
21 More detailed discussion of the 4- to 6-week studies is presented in Section 2.4.3 below (e.g., for
22 Merrick et al., 1989; Goel et al., 1992; Buben and O'Flaherty, 1985).   For this comparative
23 analysis an average is provided between two values per concentration and duration of exposure
24 for comparison with other doses and time points. As shown by the 10-day data in B6C3 F1 mice,
25 there are significant differences in response between studies of male B6C3F1 mice at the same
26 dose of TCE. This variability is similar to findings from inhalation studies of TCE in male mice
27 (Kjellstrand et al., 1983a).
28
29   As shown in Figure 4.4.2, oral TCE administration in male B6C3F1 and Swiss mice
30 appeared to induce a dose-related increase in % liver/body weight that was generally proportional
31 to the increase in magnitude of dose, though as expected, with more variability than observed for
32 a similar exercise for DCA or TCA in drinking water.   Common exposure concentrations
33 between B6C3F1 and Swiss mice were 100, 500, 1000, 1500 and 2400 mg/kg/day TCE which
34 corresponded to a 5-fold, 2-fold, 1.5-fold and 1.6-fold difference in the magnitude of dose. For
35 the data from studies in B6C3 F1 mice, there was no increase reported at 100 mg/k/day TCE but
36 between 500 and 1000, 1000 and 1500, and 1500 and 2400 mg/kg/day TCE the magnitude of
37 difference in doses matched that of the magnitude of increase in % liver/body weight (i.e., a 2.6-
38 fold, 1.4-fold, and 1.7-fold increase in liver weight was matched by a 2-fold, 1.5-fold, and 1.6-
39 fold increase in TCE exposure concentration at these exposure intervals).   However, only 10-day
40 was available for doses between 100 and 500 mg/kg in B6C3F1 mice and at the lower doses, a
41 10-day interval may have been too short for the increase in liver weight to have been fully
42 expressed. The database for the Swiss mice, which has more data from 28 and 42 days of
43 exposure, support this conclusion. At 28-42 days of exposure there was a much greater increase

1   in liver weight from TCE exposure in Swiss mice than the 10-day data in B6C3F1 mice. In
2   Figure 4.4.2, the 10-day data are included for comparative purpose for the B6C3F1 data set and
3   the Swiss and B6C3F1 datasets combined. Both the combined TCE data and that for only
4   B6C3F1 mice shows a correlation with the magnitude of dose and magnitude of % liver/body
5   weight increase. The slope of the dose response curves are both closer to that of DCA than TCA.
6   The correlation coefficients for the linear regressions presented for the B6C3F1 data is $R^2 = 0.861$
7   and for the combined data sets is $R^2 = 0.712$. Comparisons of the slopes of the dose-response
8   curves indicate that TCA is not responsible for TCE-induced liver effects. In this regression all
9   data points were treated equally although some came from several sets of data and others did not.
10   Of note is that the 2000 mg/kg TCE data point in the combined data set, which is much lower in
11   liver weight response than the other data, is from one experiment (Goel et al. 1992), from 6 mice,
12   at one time point (28 days), and one strain (Swiss). Deletion of this data point from the rest of the
13   23 used in the study results in a better fit to the data of the regression analysis.
14
15   A more direct comparison would be on the basis of dose rather than drinking water
16   concentration. The estimations of internal dose of DCA or TCA from drinking water studies have
17   been reported to vary with DeAngelo et al. reporting DCA drinking water concentrations of 1.0,
18   2.0, and 5.0 g/l to result in 90, 166, and 346 mg/kg/day, respectively. For TCA, 0.05, 0.5, 1.0,
19   2.0, and 5 g/l drinking water exposures were reported to result in 5.8 (range 3.6-8.0), 50 (range of
20   32.5 to 68), 131, 261, and 469 (range 364 to 602) mg/kg/day doses. The estimations of internal
21   dose of DCA or TCA from drinking water studies, while varying considerably (DeAngelo et al.
22   1989, 2008), nonetheless suggest that the doses of TCE used in the gavage experiments were
23   much higher than those of DCA or TCA. However, only a fraction of ingested TCE is
24   metabolized to DCA or TCA, as, in addition to oxidative metabolism, TCE is also cleared by
25   GSH conjugation and by exhalation.
26
27   While DCA dosimetry is highly uncertain (Sections 3.3 and 3.5), the mouse PBPK model,
28   described in Section 3.5 was calibrated using extensive in vivo data on TCA blood, plasma, liver,
29   and urinary excretion data from inhalation and gavage TCE exposures, and makes robust
30   predictions of the rate of TCA production. If TCA were predominantly responsible for TCE-
31   induced liver weight increases, then replacing administered TCE dose (e.g., mg TCE/kg/day) by
32   the rate of TCA produced from TCE (mg TCA/kg/day) should lead to dose-response curves for
33   increased liver weight consistent with those from directly administered TCA. Figure 4.4.3 shows
34   this comparison using the PBPK model-based estimates of TCA production for 4 TCE studies
35   from 28-42 days in the male NMRI, Swiss, and B6C3F1 mice (Kjellstrand et al. 1983b, Buben
36   and O'Flaherty 1985, Merrick et al. 1989, Goel et al. 1992) and 4 oral TCA studies in B6C3F1
37   male mice at 2 g/l or lower drinking water exposure (DeAngelo et al. 1989, 2008, Parrish et al.
38   1996, Kato-Weinstein et al. 2001) from 14-28 days of exposure. The selection of the 28-42 day
39   data for TCE was intended to address the decreased opportunity for full expression of response at
40   10 days. PBPK modeling predictions of daily internal doses of TCA in terms of mg/kg/day via
41   produced via TCE metabolism would be are indeed lower than the TCE concentrations in terms of
42   mg/kg/day given orally by gavage. The predicted internal dose of TCA from TCE exposure
43   studies are of a comparable range to those predicted from TCA drinking water studies at exposure

1   concentrations in which palability has not been an issue for estimation of internal dose. Thus
2   although the TCE data are for higher exposure concentrations, they are predicted to produce
3   comparable levels of TCA internal dose estimated from direct TCA administration in drinking
4   water.
5
6   Figure 4.4.3 clearly shows that for a given amount of TCA produced from TCE, but going
7   through intermediate metabolic pathways, the liver weight increases are substantially greater than,
8   and highly inconsistent with, that expected based on direct TCA administration. In particular, the
9   response from direct TCA administration appears to "saturate" with increasing TCA dose at a
10   level of about 1.4-fold, while the response from TCE administration continues to increase with
11   dose to 1.75-fold at the highest dose administered orally in Buben and O'Flaherty 1985) and over
12   2-fold in the inhalation study of Kjellstrand et al. (1983b). For this analysis is unlikely that strain
13   differences can account for this inconsistency in the dose-response curves. TCE-induced
14   increases in liver weight appear to be generally similar between B6C3F1 and Swiss male mice
15   (see table below) via oral exposure and between NMRI male and female mice after inhalation,
16   although the NMRI strain appeared to be more prone to TCE-induced toxicity in male mice and
17   for females to have a smaller TCE-induced liver weight increase than other strains (Kjellstrand et
18   al. 1983b). As noted previously, the difference in response between strains and between studies
19   in the same strain for TCE liver weight increases can be highly variable. Little data exists to
20   examine this issue for TCA studies although DeAngelo et al (1989) report a range of 1.16-fold to
21   1.63-fold of control % liver/body weight increase after 14 days exposure at 2 g/l TCA in the
22   Swiss-Webster, C3H, C57BL/6, and B6C3F1 strains, with differences also noted between 2
23   studies of the B6C3F1 mouse.
24
25   Furthermore, while as noted previously, oral studies appear to report a linear relationship
26   between TCE exposure concentration and liver weight induction, the inclusion of inhalation
27   studies on the basis of internal dose led to a highly consistent dose-response curve for among
28   TCE study. Therefore, it is unlikely that differing routes of exposure can explain the
29   inconsistencies in dose-response. The PBPK model predicted that matching average TCA
30   production by TCE with the equivalent average dose from drinking water-administered TCA also
31   led to an equivalent AUC of TCA in the liver. Moreover, Dees and Travis (1993) administered
32   100 to 1000 mg/kg-d TCA by gavage to male and female B6C3F1 mice for 11 days, and did not
33   observe increases in liver/body weight ratios more than 1.28-fold, no higher than those observed
34   with drinking water exposures. Finally, the dose-response consistency between TCE inhalation
35   and gavage studies argues against route of exposure significantly impacting liver weight
36   increases. Thus, no level of TCA administration appears able account for the continuing increase
37   in liver weights observed with TCE, quantitatively inconsistent with TCA being the predominant
38   metabolite responsible for TCE-induced liver weight changes. Thus, involvement of other
39   metabolites, besides TCA, is implicated as the causes of TCE-induced liver effects.
40
41   Additional analyses do, however, support a role for oxidative metabolism in TCE-induced
42   liver weight increases, and that the parent compound TCE is not the likely active moeity
43   (suggested previously by Buben and O'Flaherty 1985). In particular, the same studies are shown

1   in Figure 4.4.4 using PBPK-model based predictions of the area-under-the-curve (AUC) of TCE
2   in blood and total oxidative metabolism, which produces chloral, TCOH, DCA, and other
3   metabolites in addition to TCA. The dose-response relationship between TCE blood levels and
4   liver weight increase, while still having a significant trend, shows substantial scatter and a low R^2
5   of 0.43. On the other hand, using total oxidative metabolism as the dose metric leads to
6   substantially more consistency dose-response across studies, and a much tighter linear trend with
7   an R^2 of 0.90 (Figure 4.4.4). A similar consistency is observed using liver-only oxidative
8   metabolism as the dose metric, with R^2 of 0.86 (not shown). Thus while the slope is similar
9   between liver weight increase and TCE concentration in the blood and liver weight increase and
10   rate of total oxidative metabolism, the data are a much better fit for total oxidative metabolism.
11
12   As stated in many of the discussions of individual studies, there is a limited ability to
13   detect a statistically significant change in liver weight change in experiments that use a relatively
14   small number of animals. Many experiments have been conducted with 4-6 mice per dose group.
15   The experiments of Buben and O'Flaherty used 12-14 mice per group giving it a greater ability to
16   detect a TCE-induced dose response. In some experiments greater care was taken to document
17   and age and weight match the control and treatment groups before the start of treatment. The
18   approach taken above for the analyses of TCE, TCA and DCA uses data across several data sets
19   and gives a more robust description of these dose-response curves, especially at lower exposure
20   levels.   For example, the data from DeAngelo et al. (2008) for TCA-induced % liver/body weight
21   ratio increases in male B6C3F1 mice were only derived from 5 animals per treatment group after
22   4 weeks of exposure. The 0.05 g/l and 0.5 g/l exposure concentrations were reported to give a
23   1.09-fold and 1.16-fold of control % liver/body weight ratios which were consistent with the
24   increases noted in the cross-study database above. However, a power calculation shows that the
25   type II error, which should be > 50% and thus greater than the chances of "flipping a coin", was
26   only a 6% and 7% and therefore the designed experiment could accept a false null hypothesis.
27
28   Although the qualitative similarity to the linear dose-response relationship between DCA
29   and liver weight increases is suggestive of DCA being the predominant metabolite responsible for
30   TCE liver weight increases, due to the highly uncertain dosimetry of DCA derived from TCE, this
31   hypothesis cannot be tested on the basis of internal dose. Similarly, another TCE metabolite,
32   chloral hydrate, has also been reported to induce liver tumors in mice, however there are no
33   adequate comparative data to assess the nature of liver weight increases induced by this TCE
34   metabolite (see Section 2.5. below).   Whether its formation in the liver after TCE exposure
35   correlates with TCE-induced liver weight changes cannot be determined. Of note is the high
36   variability in total oxidative metabolism reported in mice and humans of Chapter 3 of the TCE
37   risk assessment which suggests that the correlation of total TCE oxidative metabolism with TCE-
38   induced liver effects should lead not only to a high degree of variability in response in rodent
39   bioassays which is the case (see Section 2.4.4 below) but also make detection of liver effects
40   more difficult in human epidemiological studies (see Section 4.3.2 of Chapter 3 of the TCE risk
41   assessment). What mechanisms or events are leading to liver weight increases for DCA, TCA
42   and TCE can be examined by correlations between changes in glycogen content, hepatocyte
43   volume, and evidence of polyploidization noted in short-term assays.

1   Data have been reported regarding the nature of changes the TCE and its metabolites
2   induce in the liver and are responsible for the reported increases in liver weight. Increased liver
3   weight may result from increased size or hypertrophy of hepatocytes through changes in glycogen
4   deposition, but also through increased polyploidization. Increased cell number may also
5   contribute to increased liver weight. As noted above in Section 2.4.1., hepatocellular hypertrophy
6   appeared to be related to TCE-induced liver weight changes after short-term exposures.
7   However, neither glycogen deposition, DNA synthesis, or increases in mitosis appear to be
8   correlated with liver weight increases. In particular DNA synthesis increases were similar from
9   250-1000 mg/kg and peroxisomal volume was similar between 500 mg/kg and 1500 mg/kg TCE
10 exposures after 10 days. Autoradiographs identified hepatocytes undergoing DNA synthesis in
11 "mature" hepatocytes that were in areas where polyploidization typically takes place in the liver.

Liver Weight induction as % Liver/body Weight Fold-of-control in Male B6C3F1 Mice from DCA or TCA Drinking Water Studies:

Concentration (g/l)		14 or 15 days	20 or 21 days	25 days	28 or 30 days	Mean for average of days 14-30
			Duration of Exposure			
DCA	0.1		1.02-fold			1.02-fold
	0.3	1.08-fold				1.08-fold
	0.5	1.12-fold	1.24-fold, 1.05-fold	1.16-fold	1.16-fold	1.15-fold
	1.0	1.31-fold				1.31-fold
	2.0	1.62-fold	1.46-fold, 2.01-fold	2.04-fold	1.99-fold, 1.42-fold	1.83-fold
	5.0	1.67-fold				1.67-fold
TCA	0.05				1.09-fold	1.09-fold
	0.1		0.98-fold			0.98-fold
	0.3	1.15-fold				1.15-fold
	0.5		1.13-fold		1.16-fold	1.15-fold
	1.0	1.23-fold, 1.08-fold				1.16-fold
	2.0	1.38-fold, 1.16-fold, 1.26-fold	1.33-fold			1.30-fold
	3.0				1.33-fold	1.33-fold
	5.0	1.39-fold, 1.35-fold				1.37-fold

Liver Weight induction as % Liver/body Weight Fold-of-control in Male B6C3F1 or Swiss Mice from TCE Gavage Studies:

		10 days	28 days	42 days	Mean for average of days 10-42
Concentration (mg/kg/day)					
B6C3F1					
TCE	100	1.00-fold			1.00-fold
	250	1.00-fold			1.00-fold
	500	1.20-fold, 1.06-fold			1.13-fold
	600		1.36-fold		1.36-fold
	1000	1.50-fold, 1.17-fold, 1.50-fold			1.39-fold
	1200		1.64-fold		1.64-fold
	1500	1.47-fold			1.47-fold
	2400		1.81-fold		1.81-fold
Swiss					
TCE	100			1.12-fold	1.12-fold
	200			1.15-fold	1.15-fold
	400			1.25-fold	1.25-fold
	500	1.43-fold	1.32-fold		1.38-fold
	800			1. 36-fold	1.36-fold
	1000	1.56-fold	1.41-fold		1.49-fold
	1500	1.75-fold			1.75-fold
	1600			1.63-fold	1.63-fold
	2000		1.38-fold		1.38-fold

1   2400   1.69-fold   1.69-fold
2
3   **B6C3F1 and Swiss (data sets combined)**
4
5   Mean for average of days 10-42
6
7   TCE   100   1.06-fold
8   200   1.15-fold
9   250   1.00-fold
10   400   1.25-fold
11   500   1.26-fold
12   600   1.36-fold
13   800   1.36-fold
14   1000   1.49-fold
15   1200   1.64-fold
16   1500   1.61-fold
17   1600   1.63-fold
18   2000   1.38-fold
19   2400   1.75-fold

Male B6C3F1 mice liver weight for TCA and DCA in drinking water - days 14-30
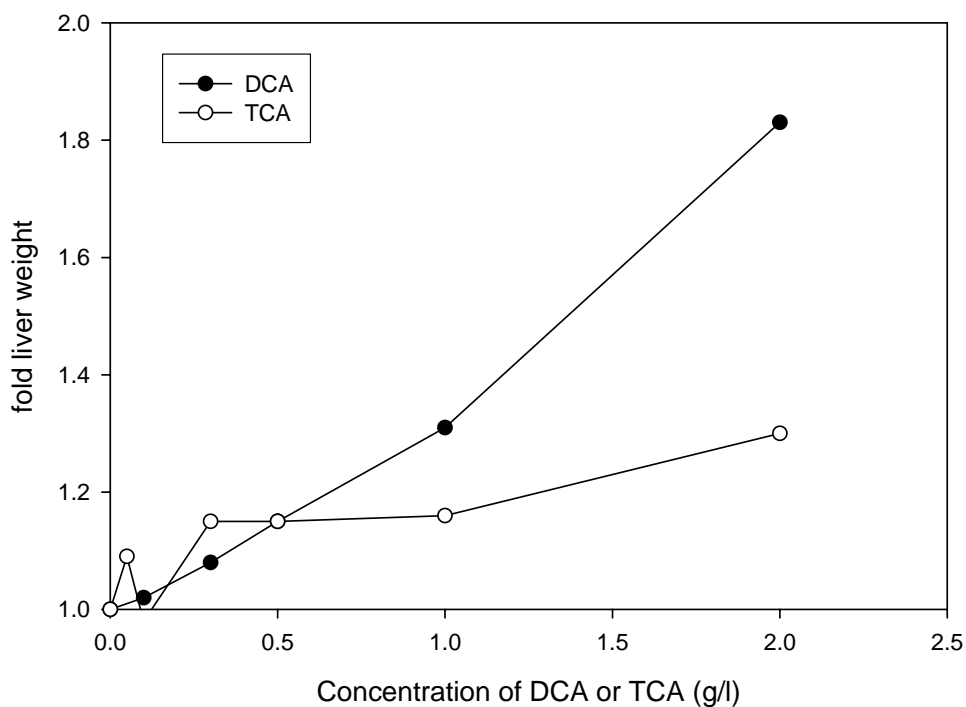
1 Figure 4.4.1 (reproduced from Section 4.4). Comparison of average fold-changes in relative
2 liver weight to control and exposure concentrations of 2 g/l or less in drinking water for TCA
3 and DCA in male B6C3F1 mice for 14-30 days (Parrish et al.,1996; Sanchez and Bull, 1990;
4 Carter et al., 1995; Kato-Weinstein et al., 2001; DeAngelo et al., 1989; 2008).

Male mice liver weight for TCE oral gavage - days 10-42
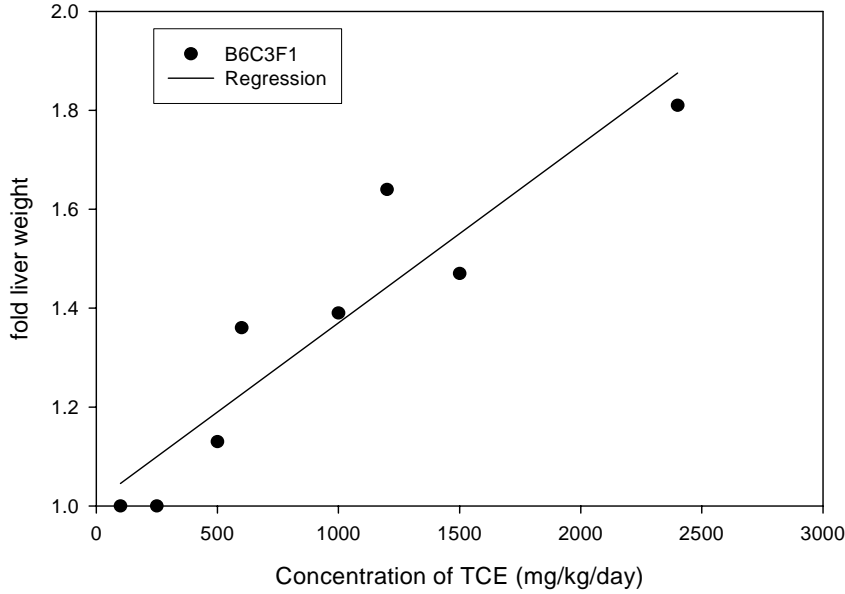
Male mice liver weight for TCE oral gavage - days 10-42
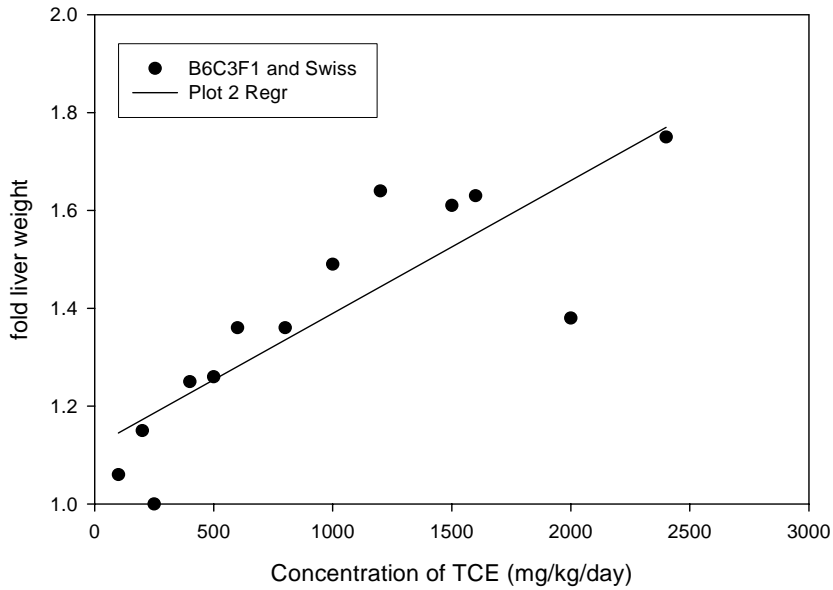
1 Figure 4.4.2 (Reproduced from Section 4.4). Comparisons of fold-changes in average relative
2 liver weight and gavage dose of (top panel) male B6C3F1 mice for 10-28 days of exposure
3 (Merrick et al., 1989; Elcombe et al., 1985; Goldsworthy and Popp 1987, Dees and Travis, 1993)
4 and (bottom panel) in male B6C3F1 and Swiss mice
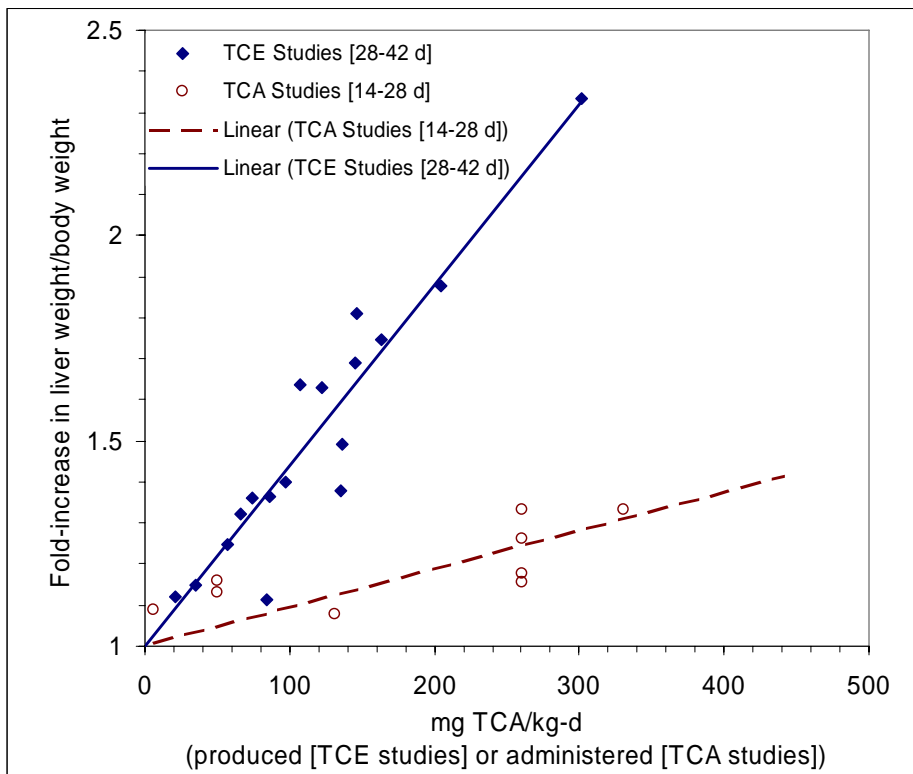
1   Figure 4.4.3 (Reproduced from Section 4.4). Comparison of fold-changes in relative liver
2   weight for data sets in male B6C3F1, Swiss, and NRMI mice between TCE studies (Kjellstrand
3   et al. 1983b, Buben and O'Flaherty 1985, Merrick et al. 1989, Goel et al. 1992) [duration 28-42
4   days] and studies of direct oral TCA administration to B6C3 F1 mice (DeAngelo et al. 1989,
5   Parrish et al. 1996, Kato-Weinstein et al. 2001, DeAngelo et al. 2008) [duration 14-28 days].
6   Abscissa for TCE studies consists of the median estimates of the internal dose of TCA predicted
7   from metabolism of TCE using the PBPK model described in Section 3.5 of the TCE risk
8   assessment. Lines show linear regression with intercept fixed at 1. All data were reported fold-
9   change in mean liver weight/body weight ratios, except for Kjellstrand et al. (1983b), with were
10 the fold-change in the ratio of mean liver weight to mean body weight. In addition, in
11 Kjellstrand et al. (1983b), some systemic toxicity as evidence by decreased total body weight
12 was reported in the highest dose group.
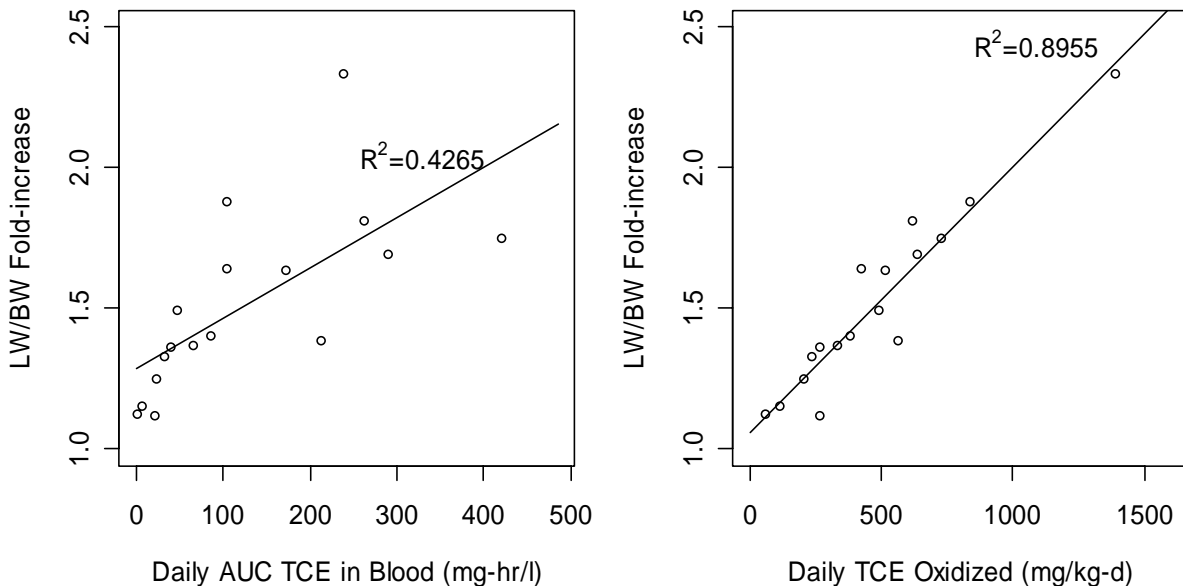
1   Figure 4.4.4 (reproduced from Section 4.4). Fold-changes in relative liver weight for data sets in
2   male B6C3F1, Swiss, and NRMI mice reported by TCE studies of duration 28-42 days
3   (Kjellstrand et al. 1983b, Buben and O'Flaherty 1985, Merrick et al. 1989, Goel et al. 1992)
4   using internal dose metrics predicted by the PBPK model described in section 3.5: (A) dose
5   metric is the median estimate of the daily AUC of TCE in blood, (B) dose metric is the median
6   estimate of the total daily rate of TCE oxidation. Lines show linear regression. Use of liver
7   oxidative metabolism as a dose metric gives results qualitatively similar to (B), with R^2=0.86.

1   By 14 days of exposure, Sanchez and Bull (1990) reported that both dose-related TCA-
2   and DCA-induced increases in liver weight were generally consistent with changing cell size
3   increases, but were not correlated with patterns of change in hepatic DNA content, incorporation
4   of tritiated thymidine in DNA extracts from whole liver, or incorporation of tritiated thymidine in
5   hepatocytes. There are conflicting reports of DNA synthesis induction in individual hepatocytes
6   for up to 14 days of DCA or TCA exposure and a lack of correlation with patterns observed for
7   this endpoint and those of whole liver thymidine incorporation. The inconsistency of whole liver
8   DNA tritiated thymidine incorporation with that reported for hepatocytes was noted by the
9   Sanchez and Bull (1990) to be unexplained. Carter et al. (1995) also report a lack of correlation
10 between hepatic DNA tritiated thymidine incorporation and labeling in individual hepatocytes in
11 male mice. Carter et al (1995) reported no increase in labeling of hepatocytes in comparison to
12 controls for any DCA treatment group from 5 to 30 days of DCA exposure. Rather than increase
13 hepatocyte labeling, DCA induced a decrease with no change reported from days 5 though 15 but
14 significantly decreased levels between days 20 and 30 for 0.5 g/l that were similar to those
15 observed for the 5 g/l exposures.
16
17 The most comparable time period between TCE, TCA and DCA results for whole liver
18 thymidine incorporation is the 10- and 14-day durations of exposure when peak tritiated
19 thymidine incorporation into individual hepatocytes and whole liver for TCA and DCA have
20 been reported to have already passed (Styles et al., 1991; Sanchez and Bull, 1990; Pereira, 1996;
21 Carter et al., 1995). Whole liver DNA synthesis was elevated over control levels by ~ 2 fold
22 after from 250 to 1000 mg/kg TCE exposure after 10 days of exposure but did not correlate with
23 mitosis (Elcombe et al., 1985; Dees and Travis,1993). After 3 weeks of exposure to TCE,
24 Laughter et al. (2004) reported in individual hepatocytes that 1% and 4.5% of hepatocytes had
25 undergone DNA synthesis in the last week of treatment for the 500 and 1000 mg/kg TCE levels,
26 respectively. More importantly, these data show that hepatocyte proliferation in TCE-exposed
27 mice at 10 days of exposure or for DCA- or TCA-exposed mice for up to 14 days of exposure is
28 confined to a very small population of cells in the liver.
29
30 In regard to cell size, although increased glycogen deposition with DCA exposure was
31 noted by Sanchez and Bull (1990), lack of quantitative analyses of that accumulation in this study
32 precludes comparison with DCA-induced liver weight gain. Although not presenting a
33 quantitative analysis, Sanchez and Bull (1990) reported DCA-treated B6C3F1 mice to have large
34 amounts of PAS staining material and Swiss-Webster mice to have similar increase despite
35 reporting differences of DCA-induced liver weight gain between the two strains. The lack of
36 concordance of the DCA-induced magnitude of increase in liver weight with that of glycogen
37 deposition is consistent with the findings for longer-term exposures to DCA reported by Kato-
38 Weinstein et al. (2001) and Pereira et al (2004) in mice (see Section 2.4.4. below). Carter et al.
39 (1995) reported that in control mice there was a large variation in apparent glycogen content and
40 also did not perform a quantitative analysis of glycogen deposition. The variability of this
41 parameter in untreated animals and the extraction of glycogen during normal tissue processing
42 for light microscopy makes quantitative analyses for dose-response difficult unless specific

1 methodologies are employed to quantitatively assess liver glycogen levels as was done by Kato-
2 Weinstein et al (2001) and Pereira et al. (2004).
3
4   Although suggested by their data, polyploidization was not examined for DCA or TCA
5 exposure in the study of Sanchez and Bull (1990). Carter et al (1995) reported that hepatocytes
6 from both 0.5 and 5 g/l DCA treatment groups were reported to have enlarged, presumably
7 polyploidy nuclei with some hepatocyte nuclei labeled in the mid-zonal area. There were
8 statistically significant changes in cellularity, nuclear size, and multinucleated cells during 30
9 days exposure to DCA. The percentage of mononucleated cells hepatocytes was reported to be
10 similar between control and DCA treatment groups at 5- and 10-day exposure. However, at 15
11 days and beyond, DCA treatments were reported to induce increases in mononucleated
12 hepatocytes. At later time periods there were also reports of DCA-induced increases nuclear
13 area, consistent with increased polyploidization without mitosis. The consistent reporting of an
14 increasing number of mononucleated cells between 15 and 30 days could be associated with
15 clearance of mature hepatocytes as suggested by the report of DCA-induced loss of cell nuclei.
16 The reported decrease in the numbers of binucleate cells in favor of mononucleate cells is not
17 typical of any stage of normal liver growth (Brodsky and Uryvaeva, 1977). The linear dose-
18 response in DCA-induced liver weight increase was not consistent with the increased numbers of
19 mononucleate cells and increase nuclear area reported from day 20 onward by Carter et al (1995).
20 Specifically, the large differences in liver weight induction between the 0.5 g/l treatment group
21 and the 5 g/l treatment groups at all times studied also did not correlate with changes in nuclear
22 size and % of mononucleate cells. Thus, DCA-induced increases in liver weight were not a
23 function of cellular proliferation, but probably included hypertrophy associated with
24 polyploidization, increased glycogen deposition and other factors.
25
26   In regard to necrosis, Elcombe et al. (1995) reported only small incidence of focal
27 necrosis in 1500 mg/kg TCE-exposed mice and no necrosis at exposures up to 1000 mg/kg for
28 10 days as did Dees and Travis (1993). Sanchez and Bull (1990) report DCA-induced localized
29 areas of coagulative necrosis both for B6C3F1 and Swiss-Webster mice at higher exposure
30 levels (1 or 2 g/l) by 14 days but not at the 0.3 g/l level or earlier time points. For TCA
31 treatment, necrosis was reported to not be associated with TCA treatment for up to 2 g/l and up
32 to 14 days of exposure. Carter et al (1995) reported that mice given 0.5 g/l DCA for 15, 20, and
33 25 days had midzonal focal cells with less detectable or no cell membranes, loss of the coarse
34 granularity of the cytoplasm, with some cells having apparent karyolysis, but for liver
35 architecture to be normal.
36
37   As for apoptosis, Both Elcombe et al. (1985) and Dees and Travis (1993) reported no
38 changes in apoptosis other than increased apoptosis only at a treatment level of 1000 mg/kg
39 TCE. Rather than increases in apoptosis, peroxisome proliferators have been suggested to
40 inhibit apoptosis as part of their carcinogenic MOA (see Section 3.4.1). However, the age and
41 species studied appear to greatly affect background rates of apoptosis. Snyder et al. (1995)
42 report that control mice were reported to exhibit apoptotic frequencies ranging from ~ 0.04 to
43 0.085%, that over the 30-day period of their study the frequency rate of apoptosis declined, and

1 suggest that this pattern is consistent with reports of the livers of young animals undergoing
2 rapid changes in cell death and proliferation. They reported rat liver to have a greater the
3 estimated frequency of spontaneous apoptosis (~ 0.1%) and therefore greater than that of the
4 mouse. Carter et al (1995) reported that after 25 days of 0.5 g/l DCA treatment apoptotic bodies
5 were reported as well as fewer nuclei in the pericentral zone and larger nuclei in central and
6 midzonal areas. This would indicate an increase in the apoptosis associated potential increases
7 in polyploidization and cell maturation. However, Snyder et al (1995) report that mice treated
8 with 0.5 g/l DCA over a 30-day period had a similar trend as control mice of decreasing
9 apoptosis with age. The percentage of apoptotic hepatocytes decreased in DCA-treated mice at
10 the earliest time point studied and remained statistically significantly decreased from controls
11 from 5 to 30 days of exposure. Although the rate of apoptosis was very low in controls,
12 treatment with 0.5g/l DCA reduced it further (~30-40% reduction) during the 30-day study
13 period. The results of this study not only provide a baseline of apoptosis in the mouse liver,
14 which is very low, but also to show the importance of taking into account the effects of age on
15 such determinations. The significance of the DCA-induced reduction in apoptosis reported in
16 this study, from a level that is already inherently low in the mouse, to account for the MOA for
17 induction of DCA-induced liver cancer is difficult to discern.
18
19 **2.4.3. Summary TCE Subchronic and Chronic Studies**
20
21   The results of longer-term (Channel et al., 1998; Toraason et al., 1999; Parrish et al.,
22 1996) studies of "oxidative stress" for TCE and its metabolites are discussed in Section 3.4.2.3.
23 Of note are the findings that the extent of increased enzyme activities associated with
24 peroxisome proliferation do not appear to correlate with measures of oxidative stress after
25 longer term exposures (Parrish et al. 1996) and single strand breaks (Chang et al., 1992).
26
27   Similar to the reports of Melnick et al (1987) in rats, Merrick et al. (1989) report that
28 vehicle (aqueous or gavage) affects TCE- induced toxicity in mice. Vehicle type made a large
29 difference in mortality, extent of liver necrosis, and liver weight gain in male and female
30 B6C3F1 mice after 4 weeks of exposure. The lowest dose used in this experiment was 600
31 mg/kg/day in males and 450 mg/kg/day in females. Administration of TCE via gavage using
32 Emulphor resulted in mortality of all of the male mice and most of the female mice at a dose in
33 corn oil that resulted in few deaths. However, use of Emulphor vehicle induced little if any
34 focal necrosis in males at concentrations of TCE in corn oil gavage that caused significant focal
35 necrosis, indicating vehicle effects.
36
37   As discussed above in Section 2.4.2, the extent of TCE-induced liver weight increases
38 was consistent between 4 and 6 weeks of exposure and between 10-day and 4 week exposure at
39 higher dose levels. In general, the reported elevations of enzymatic markers of liver toxicity and
40 results for focal hepatocellular necrosis were not consistent and did not reflect TCE dose-
41 responses observed for induction of liver weight increases (Merrick et al., 1989). Female mice
42 given corn oil and male and female mice given TCE in Emulphor were reported to have "no to
43 negligible necrosis" although they had increased liver weight from TCE exposure. Using a

1   different type of oil vehicle, Goel et al. (1992) exposed male Swiss mice to TCE in groundnut
2   oil at concentrations ranging from 500 to 2000 mg/kg for 4 weeks and reported no changes in
3   body weight up to 2000 mg/kg, although there was a 15% decrease at the highest dose, but
4   increases TCE-induced increase in % liver/body weight ratio. At a dose of 1000 and 2000
5   mg/kg, liver swelling, vacuolization, and widespread degenerative necrosis of hepatocytes was
6   reported along with marked proliferation of "endothelial cells" but no quantitation regarding the
7   extent or location of hepatocellular necrosis was reported, nor whether there was a dose-
8   response relationship in these events. They reported a TCE-related dose-response in catalase,
9   liver protein but decreased induction at the 2000 mg/kg level where body weight had decreased.
10
11   Three studies were published by Kjellstrand et al. that examined effects of TCE
12 inhalation primarily in mice using whole body inhalation chambers (Kjellstrand et al. 1981,
13 1983a, 1983b). Liver weight changes were used as the indication of TCE-induced effects. The
14 quantitative results from these experiments had many limitations due to their experimental
15 design including failure to determine body weight changes for individual animals and inability
16 to determine the exact magnitude of TCE due to concurrent oral TCE ingestion from food and
17 grooming behavior. An advantage of this route of exposure is that there were not confounding
18 vehicle effects. The results from Kjellstrand et al. (1981) are particularly limited by
19 experimental design errors but reported similar increases in liver weight gain in gerbils and rats
20 exposed at 150 ppm TCE. For rats, Kjellstrand et al (1981) do report increases in liver/body
21 weight ratios of 1.26-fold and 1.21-fold of control in male and female rat 30 days of continuous
22 TCE inhalation exposure. The unpublished report of Woolhiser et al (2006) reports 1.05-fold,
23 1.07-fold, and 1.13-fold of control % liver/body weight changes in 100 ppm, 300 ppm and 1000
24 ppm-exposure groups that are exposed for 6 hours/day, 5 days/week for 4 weeks in groups of 8
25 female CD rats. At the two highest exposure levels, body weight was reduced by TCE
26 exposure. If the 150 ppm continuous exposure concentrations of Kjellstrand are analogous to
27 750 ppm exposures using the paradigm of Woolhiser et al (2006). Therefore, the very limited
28 inhalation database for rats does indicate TCE-related increases in liver weight.
29
30   The study of Kjellstrand et al. (1983a) employed a more successful experimental design
31 that recorded liver weight changes in carefully matched control and treatment groups to
32 determine TCE-treatment related effects on liver weight in 7 strains of mice after 30 days of
33 continuous inhalation exposure at 150 ppm TCE. Individual animal body weight changes were
34 not recorded so that such an approach cannot take into account the effects of body weight
35 changes and determine a relative % liver/body weight ratio. The data presented in this report
36 was for absolute liver weight changes between treated and non-treated groups with carefully
37 matched average body weights at the initiation of exposure. A strength of the experimental
38 design is its presentation of results between duplicate experiments and thus to show the
39 differences in results between similar exposed groups that were conducted at different times.
40 This information gives a measure of variability in response with time. Mouse strain groups, that
41 did not experience TCE-induced decreased body weight gain in comparison to untreated groups
42 (i.e., DBA and "wild" type mice), represented the most accurate determination of TCE-induced
43 liver weight changes given that systemic toxicity that affects body weight can also affect liver

1   weight. The C57BL, B6CBA, and NZB groups all had at least one group out of two of male
2   mice with changes in final body weight due to TCE exposure. Only one group of NMRI mice
3   were reported in this study and that group had TCE-induced decreases in final body weight.
4   The A/sn group not only had both male groups with decreased final body weight after TCE
5   exposure (along with differences between exposed and control groups at the initiation of
6   exposure) but also a decrease in body weight in one of the female groups and thus appears to be
7   the strain with the greatest susceptibility to TCE-induced systemic toxicity. In strains of male
8   mice in which there was no TCE-induced affects on final body weight ("wild type" and DBA),
9   the influence of gender on liver weight induction and variability of the response could be more
10   readily assessed.   In "wild type" mice there was a 1.76-fold and 1.80-fold of control liver
11   weight in groups 1 and 2 for female mice, and for males a 1.84-fold and 1.62-fold of control
12   liver weight for groups 1 and 2, respectively.   For DBA mice there was a 1.87-fold and 1.88-
13   fold of control liver weight in groups 1 and 2 for female mice, and for males a 1.45-fold and
14   2.00-fold of control liver weight for groups 1 and 2, respectively. Of note, as described
15   previously, the size of the liver is under strict control in relation to body size. An essential
16   doubling of the size of the liver is a profound effect with the magnitude of liver weight size
17   increase physiologically limited..
18
19   Overall, the consistency between groups of female mice of the same strain for TCE-
20   induced liver weight gain, regardless of strain examined, was striking as was the lack of body
21   weight changes at TCE exposure levels that induced body weight changes in male mice. In the
22   absence of body weight changes, the difference in TCE-response in female mice appeared to be
23   reflective of strain and initial weight differences. Groups of female mice with higher body
24   weights, regardless of strain, generally had higher increases in TCE-induced liver weight
25   increases. For the C57BL and As/n strains, female mice starting weights were averaged 17.5 g
26   and 15.5 g, while the average liver weights were 1.63-fold and 1.64-fold of control after TCE
27   exposure, respectively.   For the B6CBA, "wild" type, DBA, and NZB female groups the
28   starting body weights averaged 22.5 g, 21.0 g, 23.0 g, and 21.0 g, while the average liver
29   weights were 1.70-fold, 1.78-fold, 1.88-fold, and 2.09-fold of control after TCE exposure,
30   respectively. The NMRI group of female mice, did not follow this general pattern and had the
31   highest initial body weight for the single group of 10 mice reported (i.e., 27 g) associated with
32   1.66-fold of control liver weight.
33
34   The results of Kjellstrand et al. (1983a) suggested that there was more variability
35   between male mice than female mice in relation to TCE-induced liver weight gain. More strains
36   exhibited TCE-induced body weight changes in male mice than female mice suggesting
37   increased susceptibility of male mice to TCE toxicity as well as more variability in response.
38   Initial body weight also appeared to be a factor in the magnitude of TCE-induced liver weight
39   induction rather than just strain. In general, the strains and groups within strain that had TCE-
40   induced body weight decreases had smaller TCE-induced increase in liver weight. Therefore,
41   only examining liver weight in males as an indication of TCE treatment effects would not be an
42   accurate predictor of strain sensitivity nor the magnitude or response at doses that also affect
43   body weight. The results from this study show that comparison of the magnitude of TCE

1   response, as measured by liver weight increases, should take into account, strain, gender, initial
2   body weight and systemic toxicity. It shows a consistent pattern of increased liver weight in
3   both male and female mice after TCE exposure of 150 ppm for 30 days.
4
5   Kjellstrand et al. (1983b) presented data in the NMRI strain of mice (a strain that
6   appeared to be more prone to TCE-induced toxicity in male mice and a smaller TCE-induced
7   increase in liver weight in female mice) after inhalation exposure of 37 to 300 ppm TCE.   They
8   used the same experimental paradigm as that reported in Kjellstrand et al (1983a) except for
9   exposure concentration.   For female mice exposed to concentrations of TCE ranging from 37
10   ppm to 300 ppm TCE continuously for 30 days, only the 300 pm group experienced a 16%
11   decrease in body weight between control and exposed animals and therefore changes in TCE-
12   induced liver weight increases were affected by changes in body weight only for that group.
13   Initial body weights in the TCE-exposed female mice were similar in each of these groups (i.e.,
14   range of 29.2 – 31.6 g, or 8%), with the exception of the females exposed to 150 ppm TCE for
15   30 days (i.e., initial body weight of 27.3 g), reducing the effects of differences in initial body
16   weight on TCE-induced liver weight induction. Exposure to TCE continuously for 30 days was
17   reported to result in a linear dose-dependent increase in liver weight in female mice with 1.06-
18   fold, 1.27-fold, 1.66-fold, and 2.14-fold of control liver weights reported at 37 ppm, 75 ppm,
19   150 ppm, and 300 ppm TCE, respectively. In male mice there were more factors affecting
20   reported liver weight increases from TCE exposure. For male mice both the 150 ppm and 300
21   ppm exposed groups experienced a 10% and 18% decrease in final body weight after TCE
22   exposure, respectively. The 37 ppm and 75 ppm groups did not have decreased final body
23   weight due to TCE exposure but varied by 12% in initial body weight. TCE-induced increases
24   in liver weight were reported to be 1.15-fold, 1.50-fold, 1.69-fold, and 1.90-fold of control for
25   37 ppm, 75 ppm, 150 ppm, and 300 ppm TCE exposure in male mice, respectively. The
26   flattening of the dose-response curve at the two highest doses is consistent with the effects of
27   toxicity on final body weight.
28
29   Kjellstrand et al. (1983b) noted that liver mass increase and the changes in liver cell
30   morphology were similar in TCE-exposed male and female mice and report that after 150 ppm
31   exposure for 30 days, liver cells were generally larger and often displayed a fine vacuolization
32   of the cytoplasm, changes in nucleoli appearance, Kupffer cells of the sinusoid to be increased
33   in cellular and nuclear size, the intralobular connective tissue was infiltrated by inflammatory
34   cells and for exposure to TCE in higher or lower concentrations during the 30 days to produce a
35   similar morphologic picture. For mice that were exposed to 150 ppm TCE for 30 days and then
36   examined 120 days after the cessation of exposure, liver weights were 1.09-fold of control for
37   TCE-exposed female mice and the same as controls for TCE-exposed male mice. However, the
38   livers were not the same as untreated liver in terms of histopathology. The authors reported that
39   "after exposure to 150 ppm for 30 days, followed by 120 days of rehabilitation, the
40   morphological picture was similar to that of the air-exposure controls except for changes in
41   cellular and nuclear sizes."   The authors did not present any quantitative data on the lesions
42   they describe, especially in terms of dose-response, and most of the qualitative description is for
43   the 150 ppm exposure level in which there are consistent reports of TCE induced body weight

1   decreases in male mice. Although stating that Kupffer cells were increased in cellular and
2   nuclear size, no differential staining was applied to light microscopy sections and used to
3   distinguish Kupffer from endothelial cells lining the hepatic sinusoid in this study. Without
4   differential staining such a determination is difficult at the light microscopic level and a question
5   remains as to whether theses are the same cells as described by Goel et al (1992) as a
6   proliferation of sinusoidal endothelial cells after exposures of 1000 mg/kg/day and 2000
7   mg/kg/day TCE exposure for 28 days in male Swiss mice. As noted in Section 2.4.2., the
8   discrepancy in DNA synthesis measures between hepatocyte examinations of individual
9   hepatocytes and whole liver measures in several reports of TCE metabolite exposure, is
10 suggestive of increased DNA synthesis in the nonparenchymal cell compartment of the liver.
11 Thus, non-parenchymal cell proliferation is suggested as an effect of subchronic TCE exposures
12 in mice without concurrent focal necrosis via inhalation studies (Kjellstrand et al., 1983b) and
13 with focal necrosis in the presence of TCE in a groundnut oil vehicle (Goel et al., 1992).
14
15   Although Kjellstrand et al. (1983b) did not discuss polyploidization, the changes in cell
16 size and especially the continued change in cell size and nuclear staining characteristics after
17 120 days of cessation of exposure are consistent with changes in polyploidization induced by
18 TCE that were suggested in studies from shorter durations of exposure (Elcombe et al., 1985;
19 Dees and Travis, 1993) and of longer durations (e.g., Buben and O'Flaherty). Of note is that in
20 the histological description provided by Kjellstrand et al (1983b), there is no mention of focal
21 necrosis or apoptosis resulting from these exposures to TCE to mice. Vacuolization is reported
22 and consistent with hepatotoxicity or lipid accumulation, which is lost during routine
23 histological slide preparation. The lack of reported focal necrosis in mice exposed through
24 inhalation is consistent with reports of gavage experiments of TCE in mice that do not use corn
25 oil as the vehicle (Merrick et al., 1989).
26
27   Buben and O'Flaherty (1985) reported the effects of TCE via corn oil gavage after six
28 weeks of exposure at concentrations ranging from 100 to 3200 mg/kg day. This study was
29 conducted with older mice than those generally used in chronic exposure assays (Male Swiss-
30 Cox outbred mice between 3 and 5 months of age). Liver weight increases, decreases in liver
31 glucose-6-phosphate (G6P) activity, increases in liver triglycerides, and increases in serum
32 glutamate-pyruvate transaminase (SGPT) activity were examined as parameters of liver toxicity.
33 Few deaths were reported during the 6-week exposure period except at the highest dose and
34 related to central nervous system depression. TCE exposure caused dose-related increases in %
35 liver/body weight with a dose as low as 100 mg/kg/day were reported to cause a statistically
36 significant increase (i.e., 112% of control). The increases in liver size were attributed to
37 hepatocyte hypertrophy, as revealed by histological examination and by a decrease in the liver
38 DNA concentration, and although enlarged, were reported to appear normal. A dose-related
39 trend toward triglyceride concentration was also noted. A dose-related decrease in glucose-6-
40 phophatase activity was reported with similar small decreases (~ 10%) observed in the TCE
41 exposed groups that did not reach statistical significance until the dose reached 800 mg/kg TCE
42 exposure. SGPT activity was not observed to be increased in TCE-treated mice except at the
43 two highest doses and even at the 2400 mg/kg dose half of the mice had normal values. The

1   large variability in SGPT activity was indicative of heterogeneity of this response between mice
2   at the higher exposure levels for this indicator of liver toxicity. Such variability of response in
3   male mice is consistent with the work of Kjellstrand et al. Thus, the results from Buben and
4   O'Flaherty (1985) suggest that hepatomegaly is a robust response that was reported to be
5   observed at the lowest dose tested, dose-related, and not accompanied by overt toxicity.
6
7   In terms of histopathology, Buben and O'Flaherty (1985) reported swollen hepatocytes
8   with indistinct borders; their cytoplasm was clumped and a vesicular pattern was apparent and
9   not simply due to edema in TCE-treated male mice. Karyorhexis (the disintegration of the
10 nucleus) was reported to be present in nearly all specimens from TCE-treated animals and
11 suggestive of impending cell death, not present in controls, and to appear at a low level at 400
12 mg/kg TCE exposure level and slightly higher at 1600 mg/kg TCE exposure level. Central
13 lobular necrosis was present only at the 1600 mg/kg TCE exposure level and at a very low level.
14 Buben and O'Flaherty report increased polyploidy in the central lobular region for both 400
15 mg/kg and 1600 mg/kg TCE and described as hepatic cells having two or more nuclei or
16 enlarged nuclei containing increased amounts of chromatin, but at the lowest level of severity or
17 occurrence. Thus, the results of this study are consistent with those of shorter-term studies via
18 gavage which report hepatocellular hypertrophy in the centralobular region, increased liver
19 weight induced at the lowest exposure level tested and at a level much lower than those inducing
20 overt toxicity, and that TCE exposure is associated with changes in ploidy.
21
22   The National Toxicology Program 13- week study of TCE gavage exposure in 10
23 F344/N rats [125 to 2000 mg/kg (males) and 62.5 to 1000 mg/kg (females)] and in B6C3F1mice
24 [375 to 6000 mg/kg] reported all rats survived the 13-week study, but males receiving 2000
25 mg/kg exhibited a 24% difference in final body weight. The study descriptions of pathology in
26 rats and mice were not very detailed and included only mean liver weights. The rats had
27 increased pulmonary vasculitis at the highest concentration of TCE and that viral titers were
28 positive for Sendai virus and no liver effects were noted for them in the study. For mice, liver
29 weights (both absolute and % liver/body weight) were reported to increase in a dose-related
30 fashion with TCE –exposure and to be increased by more than 10% in 750 mg/kg TCE- exposed
31 males and 1500 mg/kg or more TCE-exposed females. Hepatotoxicity was reported as
32 centrilobular necrosis in 6/10 males and 1/10 females exposed to 6000 mg/kg TCE and
33 multifocal areas of calcifications scattered throughout 3000 mg/kg TCE exposed male mice and
34 only a single female 6000 mg/kg dose, considered to be evidence of earlier hepatocellular
35 necrosis. One female mouse exposed to 3000 mg/kg TCE also had a hepatocellular adenoma, an
36 extremely rare lesion in female mice of this age (20 weeks). However, at the lowest dose of
37 exposure, was a consistent decrease in liver weigh in female and male mice after 13 weeks of
38 TCE exposure.
39
40   Kawamoto et al (1988) exposed rats to 2g/kg TCE subcutaneously for 15 weeks and
41 reported TCE-induced increases in liver weight. They also reported increase in cytochrome
42 P450, cytochrome b-5, and NADPH cytochrome c reductase. The difficulties in relating this
43 route of exposure to more environmentally relevant ones is discussed in Section 2.2.11.

1

2   For 2-year or lifetime studies of TCE exposure a consistent hepatocarcinogenic response
3   has been observed in mice of differing strains and genders and from differing routes of
4   exposure. However, for rat studies some studies have been confounded by mortality from
5   gavage error or the toxicity of the dose of TCE administered. In some studies, a relative
6   insensitive strain of rat has been used. However, in general it appears that the mouse is more
7   sensitive than the rat to TCE-induced liver cancer. Three studies give results the authors
8   consider to be negative for TCE-induced liver cancer in mice, but have either design and/or
9   reporting limitations, or are in strains and paradigms with apparent low ability for liver cancer
10   induction or detection.
11

12   Fukuda et al (1983) reported a 104-week inhalation bioassay in female Crj:CD-1 (ICR)
13   mice and female Crj:CD (SD) rats exposed to 0, 50, 150 and 450 ppm TCE (n=50).   There were
14   no reported incidences of mice or rats with liver tumors for controls indicative of relatively
15   insensitive strains used in the study for liver effects. While TCE was reported to induce a
16   number of other tumors in mice and rats in this study, the incidence of liver tumors was less than
17   2% after TCE exposure. Of note is the report of cystic cholangioma reported in 1 group of rats.
18

19   Henschler et al. (1980) exposed NMRI mice and WIST random bred rats to 0. 100 and
20   500 ppm TCE for 18 months (n=30). This study is limited by short duration of exposure, low
21   number of animals, and low survival in rats. Control male mice were reported to have one
22   hepatocellular carcinoma and 1 hepatocellular adenoma with the incidence rate unknown. In the
23   100 ppm TCE exposed group, 2 hepatocellular adenomas and 1 mesenchymal liver tumor were
24   reported. No liver tumors were reported at any dose of TCE in female mice or controls. For
25   male rats, only 1 hepatocellular adenomas at 100 ppm was reported. For female rats no liver
26   tumors were reported in controls, but 1 adenoma and 1 cholangiocarcinoma was reported at 100
27   ppm TCE and at 500 ppm TCE, 2 cholangioadenomas, a relatively rare biliary tumor, was
28   reported. The difference in survival in mice, did not affect the power to detect a response, as
29   was the case for rats. However, the low number of animals studied, abbreviated exposure
30   duration, and apparently low sensitivity of this paradigm (i.e., no background response in
31   controls) suggests a study of limited ability to detect a TCE carcinogenic liver response. Of note
32   is that both Fukuda et al (1983) and Henschler et al. (1980) report rare biliary cell derived
33   tumors in rats in relatively insensitive assays.
34

35   Van Duuren et al. (1979), exposed mice to 0.5 mg/mouse to TCE via gavage once a
36   week in 0.1 ml trioctanion (n= 30). Inadequate design and reporting of this study limit that
37   ability to use the results as an indicator of TCE carcinogenicity.
38

39   The NCI (1976) study of TCE was initiated in 1972 and involved the exposure of
40   Osborn-Mendel rats and B6C3F1 mice to varying concentrations of TCE. The animals were co-
41   exposed to a number of other carcinogens as exhalation as multiples studies and control animals
42   all shared the same laboratory space. Treatment duration was 78 weeks and animals received
43   TCE via gavage in corn oil at 2 doses (n= 20 for controls, but n= 50 for treatment groups). For

1 rats, the high dose was reported to result in significant mortality (i.e., 47/50 high-dose rats died
2 before scheduled termination of the study). A low incidence of liver tumors was reported for
3 controls and carbon tetrachloride positive controls in rats from this study. In B6C3F1 mice,
4 TCE was reported to increase incidence of hepatocellular carcinomas in both doses and both
5 genders of mice (~ 1170 and 2340 mg/kg for males and 870 and 1740 mg/kg for female mice).
6 Hepatocellular carcinoma diagnosis was based on histologic appearance and metastasis to the
7 lung. The tumors were described in detail and to be heterogeneous "as described in the
8 literature" and similar in appearance to tumors generated by carbon tetrachloride. The
9 description of liver tumors in this study and tendency to metastasize to the lung are similar to
10 descriptions provided by Maltoni et al (1986) for TCE-induced liver tumors in mice via
11 inhalation exposure.

13   For male rats, noncancer pathology in the NCI (1976) study was reported to include
14 increased fatty metamorphosis after TCE exposure and angiectasis or abnormally enlarged blood
15 vessels. Angiectasis can be manifested by hyperproliferation of endothelial cells and dilatation
16 of sinusoidal spaces. The authors conclude that due to mortality, "the test is inconclusive in
17 rats." They note the insensitivity of the rat strain used to the positive control of carbon
18 tetrachloride exposure.

20   The NTP (1990) study of TCE exposure in male and female F344/N rats, and B6C3F1
21 mice (500 and 1000 mg/kg for rats. and 1000 mg/kg for mice) is limited in the ability to
22 demonstrate a dose-response for hepatocarcinogenicity. There was also little reporting of non-
23 neoplastic pathology or toxicity and no report of liver weight at termination of the study.
24 However by the end of a 2-year cancer bioassay, liver tumor induction can be a significant
25 factor in any changes in liver weight. No treatment-related increase in necrosis in the liver was
26 observed in mice. A slight increase in the incidence of focal necrosis was noted for TCE-
27 exposed male mice (8% vs. 2% in control) with a slight reduction in fatty metamorphosis in
28 treated male mice (0 treated vs. 2 control animals) and in female mice a slight increase in focal
29 inflammation (29% vs. 19% of animals) and no other changes. Therefore this study did not
30 show concurrent evidence of liver toxicity but did show TCE-induced neoplasia after 2 years of
31 TCE exposure in mice. The administration of TCE was reported to cause earlier expression of
32 tumors as the first animals with carcinomas were 57 weeks for TCE-exposed animals and 75
33 weeks for control male mice.

35   The NTP (1990) study reported that TCE exposure was associated with increased
36 incidence of hepatocellular carcinoma (tumors with markedly abnormal cytology and
37 architecture) in male and female mice. Hepatocellular adenomas were described as
38 circumscribed areas of distinctive hepatic parenchymal cells with a perimeter of normal
39 appearing parenchyma in which there were areas that appeared to be undergoing compression
40 from expansion of the tumor. Mitotic figures were sparse or absent but the tumors lacked
41 typical lobular organization. Hepatocellular carcinomas had markedly abnormal cytology and
42 architecture with abnormalities in cytology cited as including increased cell size, decreased cell
43 size, cytoplasmic eosinophilia, cytoplasmic basophilia, cytoplasmic vacuolization, cytoplasmic

1   hyaline bodies and variations in nuclear appearance. Furthermore, in many instances several or
2   all of the abnormalities were present in different areas of the tumor and variations in architecture
3   with some of the hepatocellular carcinomas having areas of trabecular organization. Mitosis
4   was variable in amount and location. Therefore the phenotype of tumors reported from TCE
5   exposure was heterogenous in appearance between and within tumors.
6
7   For rats, the NTP (1990) study reported no treatment-related non-neoplastic liver lesions
8   in males and a decrease in basophilic cytological change reported from TCE-exposure in female
9   rats. The results for detecting a carcinogenic response in rats were considered to be equivocal
10   because both groups receiving TCE showed significantly reduced survival compared to vehicle
11   controls and because of a high rate (e.g., 20% of the animals in the high-dose group) of death by
12   gavage error.
13
14   The NTP (1988) study of TCE exposure in four strains of rats to "diisopropylamine-
15   stabilized TCE" was also considered inadequate for either comparing or assessing TCE-induced
16   carcinogenesis in these strains of rats because of chemically induced toxicity, reduced survival,
17   and incomplete documentation of experimental data. TCE gavage exposures of 0, 500 or 1000
18   mg/kg per day (5 days per week, for 103 weeks) male and female rats was also marked by a
19   large number of accidental deaths (e.g., for high-dose male Marshal rats 25 animals were
20   accidentally killed. Results from a 13-week study were briefly mentioned in the report and
21   indicated exposure levels of 62.5- 2000 mg/kg TCE were not associated with decreased survival
22   (with the exception of 3 male August rats receiving 2000 mg/kg TCE) and that the
23   administration of the chemical for 13 weeks was not associated with histopathological changes.
24   In regard to evidence of liver toxicity, the 2-year study of TCE exposure reported no evidence of
25   TCE-induced liver toxicity described as non-neoplastic changes ACI, August, Marshal, and
26   Osborne-Mendel rats. Interestingly, for the control animals of these four strains there was, in
27   general, a low background level of focal necrosis in the liver of both genders. In summary, the
28   negative results in this bioassay are confounded by the killing of a large portion of the animals
29   accidently by experimental error but TCE-induced overt liver toxicity was not reported.
30
31   Maltoni et al. (1986) reported the results of several studies of TCE via inhalation and
32   gavage in mice and rats. A large number of animals were used in the treatment groups but the
33   focus of the study was detection of a neoplastic response with only a generalized description of
34   tumor pathology phenotype given and limited reporting of non-neoplastic changes in the liver.
35   Accidental death by gavage error was reported not to occur in this study. In regards to effects of
36   TCE exposure on survival, "a nonsignificant excess in mortality correlated to TCE treatment
37   was observed only in female rats (treated by ingestion with the compound) and in male B6C3F1
38   mice. TCE-induced effects on body weight were reported to be absent in mice except for one
39   experiment (BT 306 bis) in which a slight non-dose correlated decrease was found in exposed
40   animals. "Hepatoma" was the term used to describe all malignant tumors of hepatic cells, of
41   different sub-histotypes, and of various degrees of malignancy and were reported to be unique or
42   multiple, and have different sizes (usually detected grossly at necropsy) from TCE exposure. In
43   regard to phenotype tumors were described as usual type observed in Swiss and B6C3F1 mice,

1 as well as in other mouse strains, either untreated or treated with hepatocarcinogens and to
2 frequently have medullary (solid), trabecular, and pleomorphic (usually anaplastic) patterns.
3 Swiss mice from this laboratory were reported to have a low incidence of hepatomas without
4 treatment (1%). The relatively larger number of animals used in this bioassay (n= 90 to 100), in
5 comparison to NTP standard assays, allows for a greater power to detect a response.
6
7   TCE exposure for 8 weeks via inhalation at 100 ppm or 600 ppm may have been
8 associated with a small increase in liver tumors in male mice in comparison to concurrent
9 controls during the life span of the animals. In Swiss mice exposed to TCE via inhalation for 78
10 weeks there a reported increase in hepatomas associated with TCE treatment that was dose-
11 related in male but not female Swiss mice. In B6C3F1 mice exposed via inhalation to TCE for
12 78 weeks, the results from one experiment indicated a greater increase in liver cancer in females
13 than male mice but in a second experiment in males there was a TCE-exposure associated
14 increase in hepatomas. Although the mice were supposed to be of the same strain, the
15 background level of liver cancer was significantly different in male mice. The finding of
16 differences in response in animals of the same strain but from differing sources has also been
17 reported in other studies for other endpoints (see Section 3.1.2.). However, for both groups of
18 male B6C3F1 mice the background rate of liver tumors over the lifetime of the mice was less
19 than 20%.
20
21   For rats, there were 4 liver angiosarcomas reported (1 in a control male rat, 1 both in a
22 TCE-exposed male and female at 600 ppm TCE for 8 weeks, and 1 in a female rat exposed to
23 600 ppm TCE for 104 weeks) but the specific results for incidences of hepatocellular
24 "hepatomas" in treated and control rats were not given. Although the Maltoni et al. (1986)
25 concluded that the small number was not treatment-related, the findings were brought forward
26 because of the extreme rarity of this tumor in control Sprague-Dawley rats, untreated or treated
27 with vehicle materials. In rats treated for 104 weeks, there was no report of a TCE treatment-
28 related increase in liver cancer in rats. This study only presented data for positive findings so it
29 did not give the background or treatment-related findings in rats for liver tumors in this study.
30 Thus, the extent of background tumors and sensitivity for this endpoint cannot be determined.
31 Of note is that the Sprague-Dawley strain used in this study was also noted in the Fukuda et al
32 (1983) study to be relatively insensitive for spontaneous liver cancer and to also be negative for
33 TCE-induced hepatocellular liver cancer induction in rats. However, like Fukuda et al (1983)
34 and Henschler et al (1980), that reported rare biliary tumors in insensitive strains of rat for
35 hepatocellular tumors, Maltoni et al, (1986) reported a relatively rare tumor type, angiosarcoma,
36 after TCE exposure in a relatively insensitive strain for "hepatomas." As noted above, many of
37 the rat studies were limited by premature mortality due to gavage error or premature mortality
38 (Henschler et al 1980; NCI 1976; NTP 1990; NTP 1988), which was reported not occur in
39 Maltoni et al (1986).
40
41   There were other reports of TCE carcinogenicity in mice from chronic exposures that
42 were focused primarily on detection of liver tumors with limited reporting of tumor phenotype
43 or non-neoplastic pathology. Herren-Freund et al. (1997) reported that male B6C3 F1 mice

1   given 40 mg/l TCE in drinking water had increased tumor response after 61 weeks of exposure.
2   However, concentrations of TCE fell by about ½ at this dose of TCE during the twice a week
3   change in drinking water solution so the actual dose of TCE the animals received was less than
4   40 mg/l. The % liver /body weight was reported to be similar for control and TCE –exposed
5   mice at the end of treatment. However, despite difficulties in establishing accurately the dose
6   received, an increase in adenomas per animal and an increase in the number of animals with
7   hepatocellular carcinomas were reported to be associated with TCE exposure after 61 weeks of
8   exposure and without apparent hepatomegaly. Anna et al., (1994) reported tumor incidences for
9   male B6C3F1 mice receiving 800 mg/kg/day TCE via gavage (5 days/week for 76 weeks). All
10 TCE-treated mice were reported to be alive after 76 weeks of treatment. Although the control
11 group contained a mixture of exposure durations (76 -134 weeks) and concurrent controls had a
12 very small number of animals, TCE-treatment appeared to increase the number of animals with
13 adenomas, the mean number of adenomas and carcinomas, but with no concurrent TCE-induced
14 cytotoxicity.

15
16 **2.4.4. Summary of Results For Sub-chronic and Chronic Effects of DCA and TCA:**
17 **Comparisons With TCE**

18
19   There are no similar studies for TCA and DCA conduced at 6 weeks and with the range
20 of concentrations examined in Buben and O'Flaherty (1985) for TCE. In general, many studies
21 of DCA and TCA have been conducted at few and high concentrations, with shortened durations
22 of exposure, and varying and low numbers of animals to examine primarily a liver tumor
23 response in mice. However, the analyses presented in Section 2.4.2 gives comparisons of
24 administered TCA and DCA dose-responses for liver weight increases for a number of studies in
25 combination as well as comparing such dose-responses to that of TCE and its oxidative
26 metabolism. As stated above, many subchronic studies of DCA and TCA have focused on
27 elucidating a relationship between dose and hypothesized events that may be indicators of
28 carcinogenic potential that have been described in chronic studies with a focus on indicators of
29 peroxisome proliferation and DNA synthesis. Many chronic studies have focused on the nature
30 of the DCA and TCA carcinogenic response in mouse liver through examination of the tumors
31 induced.

32
33   Most all of the chronic studies for DCA and TCA have been carried out in mice. As the
34 database for examination of the ability of TCE to induce liver tumors in rats includes several
35 studies that have been limited in ability determine a carcinogenic response in the liver, the
36 database for DCA and TCA in rats is even more limited. For TCA, the only available study in
37 rats (DeAngelo et al 1997) has been frequently cited in the literature to indicate a lack of
38 response in this species for TCA-induced liver tumors. Although reporting an apparent dose-
39 related increase in multiplicity of adenomas and an increase in carcinomas over control at the
40 highest dose, DeAngelo et al. (1997) use such a low number of animals per treatment group (n=
41 20-24) that the ability of this study to determine a statistically significant increase in tumor
42 response and to be able to determine that there was no treatment-related effect are limited. A
43 power calculation of the study shows that the type II error, which should be > 50%, was less

1   than 8% probability for incidence and multiplicity of all tumors at all exposure DCA
2   concentrations with the exception of the incidence of adenomas and adenomas and carcinomas
3   for 0.5 g/l treatment group (58%) in which there was an increased in adenomas reported over
4   control (15% vs. 4%) that was the same for adenomas and carcinomas combined. Therefore, the
5   designed experiment could accept a false null hypothesis and erroneously conclude that there is
6   no response due to TCA treatment. Thus, while suggesting a lower response than for mice for
7   liver tumor induction, it is inconclusive for determination of whether TCA induces a
8   carcinogenic response in the liver of rats.
9
10   For DCA, there are two reported long-term studies in rats (DeAngelo et al, 1996;
11   Richmond et al., 1995) that appear to have reported the majority of their results from the same
12   data set and which consequently were subject to similar design limitations and DCA-induced
13   neurotoxicity in this species. DeAngelo et al (1996) reported increased hepatocellular adenomas
14   and carcinomas in male F344 rats exposed for 2 years. However, the data from exposure
15   concentrations at a 5 g/l dose had to be discarded and the 2.5 g/l DCA dose had to be
16   continuously lowered during the study due to neurotoxicity. There was a DCA-induced
17   increased in adenomas and carcinomas combined reported for the 0.5 g/l DCA (24.1 % vs. 4.4%
18   adenomas and carcinomas combined in treated vs. controls) and an increase at a variable dose
19   started at 2.5 g/l DCA and continuously lowered (28.6% vs. 3.0% adenomas and carcinomas
20   combined in treated vs. controls). Only combined incidences of adenomas and carcinomas for
21   the 0.5 g/l DCA exposure group was reported to be statistically significant by the authors
22   although the incidence of adenomas was 17.2% vs. 4% in treated vs. control rats.
23   Hepatocellular tumor multiplicity was reported to be increased in the 0.5 g/l DCA group (0.31
24   adenomas and carcinomas/animal in treated vs. 0.04 in control rats) but was reported by the
25   authors to not be statistically significant. At the starting dose of 2.5 g/l that was continuously
26   lowered due to neurotoxicity, the increased multiplicity of hepatocellular carcinomas was
27   reported by the authors to be to be statistically significant (0.25 carcinomas/animals vs. 0.03 in
28   control) as well as the multiplicity of combined adenomas and carcinomas (0.36 adenomas and
29   carcinomas/animals vs. 0.03 in control rats). Issues that affect the ability to determine the nature
30   of the dose-response for this study include (1) the use of a small number of animals (n= 23,
31   n=21 and n= 23 at final sacrifice for the 2.0 g/l NaCl control, 0.05 g/l and 0.5 g/l treatment
32   groups) that limit the power of the study to both determine statistically significant responses and
33   to determine that there are not treatment-related effects (i.e. power) (2) apparent addition of
34   animals for tumor analysis not present at final sacrifice (i.e., 0.05 and 0.5 g/l treatment groups),
35   and (3) most of all, the lack of a consistent dose for the 2.5 g/l DCA exposed animals. Similar
36   issues are present for the study of Richmond et al (1995) which was conducted by the same
37   authors as DeAngelo et al (1996) and appeared to be the same data set. The Richmond et al.
38   (1995) data for the 2 g/l NaCl. 0.05 g/l DCA and 0.5 g/l DCA exposure groups were the same
39   data set reported by DeAngelo et al (1996) for these groups. Additional data was reported for
40   F344 rats administered and 2.5 g/l DCA that, due to hind-limb paralysis, were sacrificed 60
41   weeks (DeAngelo et al., 1996). Tumor multiplicity was not reported by the authors. There was
42   a small difference in reports of the results between the two studies for the same data for the 0.5
43   g/l DCA group in which Richmond et al. (1995) reported a 21% incidence of adenomas and

1   DeAngelo et al. (1996) reported a 17.2% incidence. The authors did not report any of the results
2   of DCA-induced increases of adenomas and carcinomas to be statistically significant. The same
3   issues discussed above for DeAngelo et al (1996) apply to this study. Similar to the DeAngelo
4   study of TCA in rats (DeAngelo et al. 1997) the study of DCA exposure in rats reported by
5   DeAngelo et al. (1996) and Richmond et al (1995), the use of small numbers of rats limits the
6   detection of treatment-related effects and the ability to determine whether there was no
7   treatment related effects (type II error), especially at the low concentrations of DCA exposure.
8
9   For mice the data for both DCA and TCA is much more extensive and has shown that
10 both DCA and TCA induced liver tumors in mice. Many of the studies are for relatively high
11 concentrations of DCA or TCA, have been conducted for a year or less, and have focused on the
12 nature of tumors induced to ascertain potential MOAs and to make inferences as to whether
13 TCE-induced tumors in mice are similar. As shown previously in Section 2.4.2, the dose-
14 response curves for increased liver weight for TCE administration in male mice are more similar
15 to those for DCA administration and TCE oxidative metabolism than for direct TCA
16 administration. There are two studies in male B6C3F1 mice that attempt to examine multiple
17 concentrations of DCA and TCA for 2-year studies (DeAngelo et al., 1999; DeAngelo et al.,
18 2008) at doses that do not induce cytotoxicity and attempt to relate them to subchronic changes
19 and peroxisomal enzyme induction. However, the DeAngelo et al (2008) study was carried out
20 in B6C3F1 mice that were of large size and prone to liver cancer and premature mortality
21 limiting its use for the determination of TCA-dose response in a 2-year bioassay.   One study in
22 female B6C3F1 mice describes the dose-response for liver tumor induction at a range of DCA
23 and TCA concentrations after 51 or 82 weeks (Pereira, 1996) with a focus on the type of tumor
24 each compound produced.
25
26 DeAngelo et al. (1999) conducted a study of DCA exposure to determine a dose
27 response for the hepatocarcinogenicity of DCA in male B6C3F1 mice over a lifetime exposure
28 and especially at concentrations that did not illicit cytotoxicity or were for abbreviated exposure
29 durations. DeAngelo et al. (1999) used 0.05 g/l, 0.5 g/l, 1.0 g/l, 2.0 g/l and 3.5 g/l exposure
30 concentrations of DCA in their 100-week drinking water study. The number of animals at final
31 sacrifice was generally low in the DCA treatment groups and variable (i.e., n= 50, n= 33, n= 24,
32 n= 32, n= 14, and n= 8 for control, 0.05 g/l, 0.5 g/l, 1 g/l, 2.0 g/l and 3.5 g/l DCA exposure
33 groups). It was apparent that animals that died unscheduled deaths between weeks 79 and 100
34 were included in data reported for 100 weeks. Although the authors did not report how many
35 animals were included in the 100-week results, it appeared that the number was no greater than
36 1 for the control, 0.05, and 0.5 exposure groups and varied between 3 and 7 for the higher DCA
37 exposure groups. The multiplicity or number of hepatocellular carcinomas/animals was
38 reported to be significantly increased over controls in a dose-related manner at all DCA
39 treatments including 0.05 g/l DCA, and a NOEL reported not to be observed by the authors (i.e.,
40 0.28, 0.58, 0.68, 1.29, 2.47, and 2.90 hepatocellular carcinomas/ animal for control, 0.05, 0.5,
41 1.0, 2.0, and 3.5 g/l DCA). Between the 0.5 g/l and 3.5 g/l exposure concentrations of DCA the
42 magnitude of increase in multiplicity was similar to the increases in magnitude in dose. The
43 incidence of hepatocellular carcinomas were reported to be increased at all doses as well but not

1   reported to be statistically significant at the 0.05 g/l exposure concentration. However given
2   that the number of mice examined for this response (n= 33), the power of the experiment at this
3   dose was only 16.9% to be able to determine that there was not a treatment related effect. The
4   authors did not report the incidence or multiplicity of adenomas for the 0.05 g/l exposure group
5   in the study and neither did they report the incidence or multiplicity of adenomas and
6   carcinomas in combination. For the animals surviving from 79 to 100 weeks of exposure, the
7   incidence and multiplicity of adenomas peaked at 1 g/l while hepatocellular carcinomas
8   continued to increase at the higher doses. This would be expected where some portion of the
9   adenomas would either regress or progress to carcinomas at the higher doses.
10
11   DeAngelo et al (1999) reported that peroxisome proliferation was significantly increased
12   at 3.5 g/l DCA only at 26 weeks, not correlated with tumor response, and to not be increased at
13   either 0.05 g/l or 0.5 g/l treatments. The authors concluded that DCA-induced carcinogenesis
14   was not dependent on peroxisome proliferation or chemically sustained proliferation, as
15   measured by DNA synthesis. DeAngelo et al (1999) reported not only a dose-related increase in
16   DCA-induced liver tumors but also a decrease in time-to-tumor associated with DCA exposure
17   at the lowest levels examined. In regards to cytotoxicity there appeared to be a treatment but not
18   dose-related increase in hepatocellular necrosis that did not involve most of the liver from 1 g/l
19   to 3.5 g/l DCA exposures for 26 weeks of exposure that decreased by 52 weeks with no necrosis
20   observed at the 0.5 g/l DCA treatment for any exposure period.
21
22   Hepatomegaly was reported to be absent by 100 weeks of exposure at the 0.05g/l and 0.5
23   g/l exposures while there was an increase in tumor burden reported. However, slight
24   hepatomegaly was present by 26 weeks in the 0.5 g/l group and decreased with time.   Not only
25   did the increase in multiplicity of hepatocellular carcinomas increase proportionally with DCA
26   exposure concentration after 79-100 weeks of exposure, but so did the increases in % liver/body
27   weight. DeAngelo et al. (1999) presented a figure comparing the number of hepatocellular
28   carcinomas/animal at 100 weeks compared with the % liver/body weight at 26 weeks that
29   showed a linear correlation ($r^2 = 0.9977$) while peroxisome proliferation and DNA synthesis did
30   not correlate with tumor induction profiles. The proportional increase in liver weight with DCA
31   exposure was also reported for shorter durations of exposure as noted in Section 2.4.2.   The
32   findings of the study illustrates the importance of examining multiple exposure levels at lower
33   concentrations, at longer durations of exposure and with an adequate number of animals to
34   determine the nature of a carcinogenic response. Although Carter et al. (1995) suggested that
35   there is evidence of DCA-induced cytotoxicity (e.g., loss of cell membranes and apparent
36   apoptosis) at higher levels, the 0.5 g/l exposure concentration has been shown by DeAngelo et al
37   (1999) to increase hepatocellular tumors after 100 weeks of treatment without concurrent
38   peroxisome proliferation or cytotoxicity in mice.
39
40   As noted in detail in Section 2.3.2.13, DeAngelo et al. (2008) exposed male B6C3F1
41   mice to neutralized TCA in drinking water to male B6C3 F1 mice in three studies. Rather than
42   using 5 exposure levels that were generally 2-fold apart, as was done in DeAngelo et al. (1999)
43   for DCA, DeAngelo et al (2008) studied only 3 doses of TCA that were an order of magnitude

1   apart which limits the elucidation of the shape of the dose-response curve. In addition
2   DeAngelo et al. (2008) contained 2 studies, each conducted in a separate laboratories, for the
3   104-week data so that the two lower doses were studied in one study and the highest dose in
4   another. The first study was conducted using 2 g/l NaCl, or 0.05, 0.5, or 5 g/l TCA in drinking
5   water for 60 weeks (Study #1) while the other two were conducted for a period of 104 weeks
6   (Study #2 with 2.5 g/l neutralized acetic acid or 4.5 g/l TCA exposure groups and Study #3 with
7   deionized water, 0.05 g/l TCA and 0.5 g/l TCA exposure groups). In the studies reported in
8   DeAngelo et al (2008) a small number of animals has been used for the determination of a tumor
9   response (~ n= 30 at final necropsy), but for the data for liver weight or PCO activity at interim
10 sacrifices the number was even smaller (n= 5). The % liver/body weight changes at 4 weeks in
11 Study #1 have been included in the analysis for all TCA data in Section 2.4.2, and are consistent
12 with that data. Although there was a 10-fold difference in TCA exposure concentration, there
13 was a 9%, 16% and 35% increase in liver weight over control for the 0.05g/l 0.5 g/l and 5 g/l
14 TCA exposures. PCO activity varied 2.7-fold as baseline controls but the increase in PCO
15 activity at 4 weeks was 1.3-fold, 2.4-fold, and 5.3-fold of control for the 0.05 g/l, 0.5 g/l and 5
16 g/l TCA exposure groups in Study #1. The incidence data for adenomas observed at 60 weeks
17 was 2.1-fold, 3.0-fold and 5.4-fold of control values and the fold increases in multiplicity were
18 similar after 0.05 g/l, 0.5 g/l and 5.0 g/l TCA. Thus, in general the dose-response for TCA-
19 induced liver weight increases at 4 weeks was similar to the magnitude of induction of
20 adenomas at 60 weeks. Such a result is more consistent with the ability of TCA to induce tumors
21 and increases in liver weight at low doses with little change with increasing dose as shown by
22 this study and the combined data for TCA liver weight induction by administered TCA
23 presented in Section 2.4.2.
24
25   While the 104-week data from Study's #2 and #3 could have been more valuable for
26 determination of the dose-response as it would have allowed enough time for full tumor
27 expression, serious issues are apparent for Study #3 which was reported to have a 64%
28 incidence rate of adenomas and carcinomas for controls while that of Study #2 was 12%. As
29 stated in Section 2.3.2.13, the mice in Study #3 were of larger size than those of either Study #1
30 or #2 and the large background rate of tumors reported is consistent with mice of these size
31 (Leakey et al., 2003b). However, the large background rate and increased mortality for these
32 mice limit their use for determining the nature of the dose-response for TCA liver
33 carcinogenicity. Examination of the data for treatment groups shows that there was no
34 difference in any of the results between the 0.5 g/l (Study #3) and 5 g/l (Study #2) TCA
35 exposure groups (i.e., adenoma, carcinoma, and combinations of adenoma and carcinoma
36 incidence and multiplicity) for 104 weeks of exposure. For these same exposure groups, but at
37 60 weeks of exposure (Study #1), there was a 2-fold increase in multiplicity for adenomas, and
38 for adenomas and carcinomas combined between the 0.5 and 5.0 g/l TCA exposure groups. At
39 the two lowest doses of 0.05 g/l and 0.5 g/l TCA from Study #3 in the large tumor prone mice,
40 the differences in the incidences and multiplicities for all tumors were 2-fold at 104 weeks.
41 These results are consistent with (1) the two highest exposure levels reaching a plateau of
42 response after a long enough duration of exposure for full expression of the tumors (i.e., ~ 90%
43 of animals having liver tumors at the 0.5 g/l and 5 g/l exposures) with the additional tumors

1   observed in a tumor-prone paradigm. Thus, without use of the 0.05 g/l and 0.5 g/l TCA data
2   from Study #3, only the 4.5 g/l TCA data from study #2 can be used for determination of the
3   TCA cancer response in a 2-year bioassay.
4
5   To put the 64% incidence data for carcinomas and adenomas reported in DeAngelo et al.
6   (2008) for the control group of Study #3 in context, other studies cited in this review for male
7   B6C3F1 mice show a much lower incidence in liver tumors with: (1) NCI (1976) study of TCE
8   reporting a colony control level of 6.5% for vehicle and 7.1% incidence of hepatocellular
9   carcinomas for untreated male B6C3F1 mice (n= 70 – 77) at 78 weeks, (2) Herren-Freund et al
10 (1987) reporting a 9% incidence of adenomas in control male B6C3F1 mice with a multiplicity
11 of 0.09 ± 0.06 and no carcinomas (n= 22) at 61 weeks, (3) NTP (1990) reporting an incidence
12 of 14.6% adenomas and 16.6% carcinomas in male B6C3F1 mice after 103 weeks (n= 48), and
13 (4) Maltoni et al (1986) reporting that B6C3F1 male mice from the "NCI source" had a 1.1%
14 incidence of "hepatoma" (carcinomas and adenomas) and those from "Charles River Co." had a
15 18.9% incidence of "hepatoma" during the entire lifetime of the mice (n= 90 per group).   The
16 importance of examining an adequate number of control or treated animals before confidence
17 can be placed in those results in illustrated by Anna et al (1994) in which at 76 weeks 3/10
18 control male B6C3F1 mice that were untreated and 2/10 control animals given corn oil were
19 reported to have adenomas but from 76 to 134 weeks, 4/32 mice were reported to have
20 adenomas (multiplicity of 0.13 ± 0.06) and 4/32 mice were reported to have carcinomas
21 (multiplicity of 0.12 ± 0.06). Thus, the reported combined incidence of carcinomas and
22 adenomas of 64% reported by DeAngelo et al (2008) for the control mice of Study # 3, not only
23 is inconsistent and much higher than those reported in Studies #1 and #2, but also much higher
24 than reported in a number of other studies of TCE .
25
26   Trying to determine a correspondence with either liver weight increases or increases in
27 PCO activity after shorter periods of exposure will be depend whether data reported in Study #3
28 in the 104 week studies can be used. DeAngelo et al. (2008) report a regression analyses that
29 compare "percent of hepatocellular neoplasia," indicated by tumor multiplicity, with TCA
30 dose, represented by estimations of the TCA dose in mg/kg/day, and with PCO activity for the
31 60-week and 104-week data. Whether adenomas and carcinomas combined or individual tumor
32 type were used in these analysis was not reported by the authors. Concerns arise also from
33 comparing PCO activity at the end of the experiments, when there was already a significant
34 tumor response, rather than at earlier time points. Such PCO data may not be useful as an
35 indicator key event in tumorigenesis when tumors are already present. In addition regression
36 analyses of this data are difficult to interpret because of the dose spacing of these experiments as
37 the control and 5 g/l exposure levels will basically determine the shape of the dose response
38 curve. The 0.05 g/l and 0.5 g/l exposure levels are close to the control value in comparison to
39 the 5 g/l exposure level, the dose response appears to be linear between control and the 5.0 g/l
40 value with the two lowest doses not affectly changing the slope of the line (i.e., "leveraging" the
41 regression). Thus, the value of these analyses is limited by (1) use of data from Study # 3 in a
42 tumor prone mouse that is not comparable to those used in Studies #1 and #2, (2) the
43 appropriateness of using PCO values from later time points and the variability in PCO control

1   values (3) the uncertainty of the effects of palatability on the 5 g/l TCA results which were
2   reported in one study to reduce drinking water consumption, and (4) the dose-spacing of the
3   experiment.
4
5   DeAngelo et al (2008) attempt to identify a NOEL for tumorigenicity using tumor
6   multiplicity data and estimated TCA dose. However, it is not an appropriate descriptor for these
7   data, especially given that "statistical significance" of the tumor response is the determinant
8   used by the authors to support the conclusions regarding a dose in which there is no TCA-
9   induced effect. Due to issues related to the appropriateness of use of the concurrent control in
10   Study #3, only the 60-week experiment (i.e., study # 1) is useful for the determination of tumor
11   dose-response. Not only is there not allowance for full expression of a tumor response at the 60-
12   week time point but a power calculation of the 60-week study shows that the type II error, which
13   should be > 50% and thus greater than the chances of "flipping a coin", was 41% and 71% for
14   incidence and 7% and 15% for multiplicity of adenomas for the 0.05 g/l and 0.5 g/l TCA
15   exposure groups. For the combination of adenomas and carcinomas, the power calculation was
16   8% and 92% for incidence and 6% and 56% for multiplicity at 0.05 g/l and 0.5 g/l TCA
17   exposure. Therefore, the designed experiment could accept a false null hypothesis, especially in
18   terms of tumor multiplicity, at the lower exposure doses and erroneously conclude that there is
19   no response due to TCA treatment.
20
21   Pereira (1996) examined the tumor induction in female B6C3 F1 mice and demonstrate
22   that foci, adenoma, and carcinoma development in mice are dependent on duration of exposure,
23   or period of observation in the case of controls, for full expression of a carcinogenic response.
24   In control female mice a 360- vs. 576-day observation period showed that at 360 days no foci or
25   carcinomas and only 2.5% of animals had adenomas whereas by 576 days of observation, 11%
26   had foci, 2% adenomas, and 2% had carcinomas. For DCA and TCA treatments, foci,
27   adenomas, and carcinoma incidence and multiplicity did not reach full expression until 82
28   weeks at the 3 doses employed (2.58 g/l DCA, 0.86 g/l DCA, 0.26 g/l DCA, 3.27 g/l TCA, 1.1.0
29   g/l TCA, and 0.33 g/l TCA). Although the numbers of animals were relatively low and variable
30   at the two highest doses (18-28 mice) there were 50-53 mice studied at the lowest dose level and
31   90 animals studied in the control group. The results of Pereira (1996) show that not only were
32   the incidence of mice with foci, adenoma, and carcinomas greatly increased with duration of
33   exposure, but that concentration also affected the nature and magnitude of the response in
34   female mice. At 2.86 g/l, 0.86 g/l, 0.26 g/l DCA exposures and controls, after 82 weeks the
35   incidence of adenomas in female B6C3 F1 mice was reported to be 84.2%, 25.0%, 6.0% and
36   2.2%, respectively, and carcinomas to be 26.3 %, 3.6%, 0%, and 2.2%, respectively. For the
37   multiplicity or number of tumors/animal at these same exposure levels of DCA, the multiplicity
38   was reported to be 5.58, 0.32, 0.06 and 0.02 adenomas/animal, and 0.37, 0.04, 0 and 0.02
39   carcinomas/animal. Thus, for DCA exposure in female mice, for ~ 3-fold increases in DCA
40   exposure concentration, after 82 weeks of exposure there was a similar magnitude of increase in
41   adenomas incidence with much greater increases in multiplicity. For hepatocellular carcinoma
42   induction, there was no increase in the incidence or multiplicity or carcinomas between the
43   control and 0.33 g/l DCA dose. At 3.27 g/l, 1.10 g/l. 0.33 g/l TCA and controls, after 82 weeks

1   the incidence of adenomas in female B6C3F1 mice was reported to be 38.9%, 11.1%, 7.6%, and
2   2.2%, respectively, and carcinomas to be 27.8%, 18.5%, 0 % and 2.2%, respectively. At these
3   same exposure levels of TCA, the multiplicity was reported to be 0.61, 0.11, 0.08, and 0.02
4   adenomas/animal, and 0.39, 0.22, 0, and 0.02 carcinomas/animal, respectively. Thus for TCA,
5   the incidences of adenomas were lower at the two highest doses than DCA and the ~3-fold
6   differences in dose between the two lowest doses only resulted in ~ 50% increase in incidences
7   of adenomas. For incidence of carcinomas the ~ 3-fold difference in dose between the two
8   highest doses only resulted in ~ 50% increase in carcinoma incidence. A similar pattern was
9   reported for multiplicity after TCA exposure. Foci were also examined and, in general., were
10 similar to adenomas regarding incidence and multiplicity. Thus, the dose-response curve for
11 tumor induction in female mice differed between DCA and TCA after 82 weeks of exposure
12 with TCA having a much less steep dose-response curve than DCA. This is consistent with the
13 pattern of liver weight increases reported for male B6C3F1 mice in Section 2.4.2.
14
15   DeAngelo et al. (1999) report a linear increase in incidence and multiplicity of
16 hepatocellular carcinomas that is proportional to dose and as well as proportional to the
17 magnitude of liver weight increase from subchronic exposure to DCA. However the studies of
18 DeAngelo et al. (2008) and Pereira (1996) are suggestive that TCA induced increase in tumor
19 incidence are less proportional to increases in dose as are liver weight increases from subchronic
20 exposure. Given that TCE subchronic exposure also induced an increase in liver weight that
21 was proportional to dose (i.e., similar to DCA but not TCA), it is of interest as to whether the
22 dose-response for TCE induced liver cancer in mice was similar. The database for TCE, while
23 consistently showing a induction of liver tumors in mice, is very limited for making inferences
24 regarding the shape of the dose-response curve. For many of these experiments multiplicity was
25 not given only liver tumor incidence.   NTP (1990), Bull et al (2002), Anna et al. (1994)
26 conducted gavage experiments in which they only tested one dose of ~ 1000 mg/kg/day TCE.
27 NCI (1976) tested 2 doses that were adjusted during exposure to an average of 1169 mg/kg/day
28 and 2339 mg/kg/day in male mice with only 2-fold dose spacing in only 2 doses tested. Maltoni
29 et al (1988) conducted inhalation experiments in 2 sets of B6C3F1 mice and one set of Swiss
30 mice at 3 exposure concentrations that were 3-fold apart in magnitude between the low and mid-
31 dose and 2-fold apart in magnitude between the mid- and high-dose. However for one
32 experiment in male B6C3F1 mice, the mice fought and suffered premature mortality and for two
33 the experiments in B6C3F1 mice, although using the same strain, the mice were obtained from
34 differing sources with very different background liver tumor levels. For the Maltoni et al (1988)
35 study a general descriptor of "hepatoma" was used for liver neoplasia rather than describing
36 hepatocellular adenomas and carcinomas so that comparison of that data with those from other
37 experiments is difficult. More importantly, while the number of adenomas and carcinomas may
38 be the same between treatments or durations of exposure, the number of adenomas may decrease
39 as the number of carcinomas increase during the course of tumor progression. Such information
40 is lost by using only a hepatoma descriptor. Maltoni et al (1988) did not report an increase over
41 control for 100 ppm TCE for the Swiss group and one of the B6C3F1 groups and only a slight
42 increase (1.12-fold) in the second B6C3F1 group. At 300 ppm TCE exposure, the incidences of
43 hepatoma were 2-fold of control values for the Swiss, 4-fold of control for group of B6C3F1

1 mice, and 1.6-fold of control for the other group of B6C3F1 mice. At 600 ppm TCE the
2 incidences of hepatoma were 3.3-fold of control for the Swiss group, 6.1-fold of control for one
3 group of B6C3F1 mice, and 1.2-fold for the other group of B6C3F1 mice. Thus, for each group
4 of TCE exposed mice in the Maltoni et al. (1988) inhalation study, the background levels of
5 hepatomas and the shape of the dose-response curve for TCE-hepatoma induction were variable.
6 However, an average of the increases, in terms of fold of control, between the 3 experiments
7 gives a ~ 2.9- fold increase between the low- and mid-dose (100 ppm and 300 ppm) and ~ 1.4-
8 fold increase between the mid- and high-dose (300ppm and 600 pm) groups. Although such a
9 comparison obviously has a high degree of uncertainty associated with it, it suggests that the
10 magnitude of TCE-induced hepatoma increases over control is similar to the 3-fold and 2-fold
11 difference in the magnitude of exposure concentrations between these doses. Therefore the
12 increase in TCE-induced liver tumors would roughly proportional to the magnitude of exposure
13 dose. This result would be similar to the result for the concordance of the increases in liver
14 weight and exposure concentration observed 28-42 day exposures to TCE (see Section 2.4.2)
15 using oral data from B6C3F1 and Swiss mice, and inhalation data from NMRI mice. The
16 available inhalation data for TCE induced liver weight dose-response is from one study in a
17 strain derived from Swiss mice (Kjellstrand et al., 1983b) and was conducted in male and female
18 mice with comparable doses of 75 ppm and 300 ppm TCE. However, male mice of this strain
19 exhibited decreased body weight at the 300 ppm level which can affect %liver/body weight
20 increases. The magnitude of TCE-induced increases in liver weight between the 75 ppm and
21 300 ppm exposures were ~ 1.80-fold for males (1.50 vs. 1.90-fold of control liver weights) and
22 4.2-fold for females (1.27-fold vs. 2.14-fold of control liver weight) in this strain. Female mice
23 were examined in one study each of Swiss and B6C3F1 mice by Maltoni et al. (1988). Both the
24 Swiss and B6C3F1 studies reported increases in incidences of hepatomas over controls only at
25 the 600 ppm TCE level in female mice indicating less of a response than males. Similarly, the
26 Kjellstrand et al (1983b) data also showed less of a response in females compared to males in
27 terms TCE induction of liver weight at the 37 to 150 ppm range of exposure in NMRI strain.
28 While the data for TCE dose-response of liver tumor induction is very limited, it is suggestive of
29 a correlation of TCE-induced increases in liver weight correlating liver tumor induction with a
30 pattern that is dissimilar to that of TCA.
31
32   Of those experiments conducted at ~ 1000 mg/kg/day gavage dose of TCE in male
33 B6C3F1 mice for at least 79 weeks (Bull et al., 2002; NCI, 1976; Anna et al., 1994, NTP, 1990)
34 the control values were conducted in varying numbers of animals (some as low as n=15, i.e.,
35 Bull et al., 2002) and with varying results. The incidence of hepatocellular carcinomas ranged
36 from 1.2% to 16.7% (NCI, 1976; Anna et al., 1994, NTP, 1990) and the incidence of adenomas
37 ranged from 1.2% to 14.6% (Anna et al., 1994, NTP, 1990) in control B6C3F1 mice. After ~
38 1000 mg/kg/day TCE treatment, the incidence of carcinomas ranged from 19.4% to 62% (Bull
39 et al., 2002; NCI, 1976; Anna et al., 1994, NTP, 1990) with 3 of the studies (NCI, 1976; Anna et
40 al., 1994, NTP, 1990) reporting a range of incidences between 42.8% to 62.0%). The incidence
41 of adenomas ranged from 28% to 66.7% (Bull et al., 2002; Anna et al., 1994; NTP, 1990).
42 These data are illustrative of the variability between experiments to determine the magnitude
43 and nature of the TCE response in the same gender (male), strain (B6C3F1), time of exposure

1   (3/4 studies were for 76-79 weeks and 1 for 2 years duration), and roughly the same dose (800 –
2   1163 mg/kg/day TCE). Given, that the TCE-induced liver response, as measured by liver
3   weight increase, is highly correlated with total oxidative metabolism to a number of agents that
4   are hepatoactive agents and hepatocarcinogens, the variability in response from TCE exposure
5   would be expected to be greater than studies of exposure to a single metabolite such as TCA or
6   DCA.
7
8   Caldwell et al (2007b) have commented on the limitations of experimental paradigms
9   used to study liver tumor induction by TCE metabolites and show that 51-week exposure
10   duration has consistently produced a tumor response for these chemicals, but with greater lesion
11   incidence and multiplicity at 82 weeks. As reported by DeAngelo et al. (1999) and Pereira
12   (1996), full expression of tumor induction in the mouse does not occur until 78 to 100 weeks of
13   DCA or TCA exposure, especially at lower concentrations. Thus, use of abbreviated exposure
14   durations and concurrently high exposure concentrations limits the ability of such experiments
15   to detect a treatment-related effect with the occurrence of additional toxicity not necessarily
16   associated with tumor-induction. Caldwell et al (2007b) present a table that shows that the
17   differences in the ability of the studies to detect treatment-related effects could also be attributed
18   to a varying and low number of animals in some exposure groups and that because of the low
19   numbers of animals tested at higher exposures, the power to detect a statistically significant
20   change is very low and in fact for many of the endpoints is considerably less than "50%
21   chance." The table below from Caldwell et al. (2008b) illustrates the importance of
22   experimental design and the limitations in many of the studies in the TCE metabolite database.

Table II. Power calculations* for experimental design described in text, using Pereira et al. as an example.

Exposure concentration† in female B6C3F1 mice (Pereira, 1996 and Pereira and Phelps, 1996)	Number of animals	Power calculation for Foci	Power calculation for Adenomas	Power calculation for Carcinomas
20.0 mmol/L NaCl (control) (82 wk)	90	Null hypothesis	Null hypothesis	Null hypothesis
2.58 g/L DCA (82 wk)	19	0.03	0.03	0.13
0.86 g/L DCA (82 wk)	28	0.74	0.20	0.91
0.26 g/L DCA (82 wk)	50	0.99	0.98	-
3.27 g/L TCA (82 wk)	18	0.15	0.09	0.14
1.10 g/L TCA (82 wk)	27	0.60	0.64	0.3
0.33 g/L TCA (82 wk)	53	0.93	0.91	-

1   †Conversion of mmol/L to g/L from the original reports of Pereira (1996) and Pereira and Phelps (1996) is as follows:
2   20.0 mmol/L DCA = 2.58 g/L, 6.67 mmol/L DCA = 0.86 g/L, 2.0 mmol/L = 0.26 g/L, 20.0 mmol/L TCA = 3.27 g/L,
3   6.67 mmol/L TCA = 1.10 g/L, 2.0 mmol/L TCA = 0.33 g/L.
4
5   *The power calculations represent the probability of rejecting the null hypothesis when in fact the alternate hypothesis
6   is true for tumor multiplicity (i.e., the total # of lesions/# animals). The higher the power number calculated, the more
7   confidence we have in the null hypothesis. Assumptions made included: normal distribution for the fraction of tumors
8   reported, null hypothesis represents what we expected the control tumor fraction to be, the probability of a Type I error
9   was set to 0.05, and the alternate hypothesis was set to four times the null hypothesis value.

1   Bull et al. (1990) examined male and female B6C3F1 mice (age 37 days) exposed from
2   15 to 52 weeks to neutralized DCA and TCA (1 g/l or 2 g/l) but tumor data was not suitable for
3   dose response. They reported effects of DCA and TCA exposure on liver weight and %
4   liver/body changes that gave a pattern of hepatomegaly generally consistent with short-term
5   exposure studies. Only 10 female mice were examined at 52 weeks but the female mice were
6   reported to be as responsive as males at the exposure concentration tested. After 37 weeks of
7   treatment and then a cessation of exposure for 15 weeks, liver weights % liver/body weight were
8   reported to be elevated over controls which Bull et al. (1990) partially attribute the remaining
9   increases in liver weight to the continued presence of hyperplastic nodules in the liver.
10   Macroscopically, livers treated with DCA were reported to have multi-focal areas of necrosis and
11   frequent infiltration of lymphocytes on the surface and an interior of the liver. For TCA-treated
12   mice, similar necrotic lesions were reported but at such a low frequency that they were similar to
13   controls. Marked cytomegaly was reported from exposure to either 1 or 2 g/l DCA throughout
14   the liver. Cell size was reported to be increased from TCA and DCA treatment with DCA
15   producing the greatest change. The 2 g/l TCA exposures were observed to have increased
16   accumulations of lipofuscin but no quantitative analysis was done. Photographs of light
17   microscopic sections, that were supposed to be representative of DCA and TCA treated livers at
18   2g/l, showed such great hepatocellular hypertrophy from DCA treatment that sinusoids were
19   obscured. Such a degree of cytomegaly could have resulted in reduction of blood flow and
20   contributed to focal necrosis observed at this level of exposure.
21
22   As discussed in Sections 3.2 and 3.4.2.1, glycogen accumulation has been described to be
23   present in foci in both humans and animals as a result from exposure to a wide variety of
24   carcinogenic agents and predisposing conditions in animals and humans. Bull et al (1990)
25   reported that glycogen deposition was uniformly increased from 2 g/l DCA exposure with
26   photographs of TCA exposure showing slightly less glycogen staining than controls. However,
27   the abstract and statements in the paper suggest that there was increased PAS positive material
28   from TCA treatment that has caused confusion in the literature in this regard. Kato-Weinstein et
29   al. (2001) reported that in male B6C3F1 mice exposed to DCA and TCA, the DCA treatment
30   increased glycogen and TCA decreased glycogen content of the liver by using both chemical
31   measurement of glycogen in liver homogenates and by using ethanol-fixed sections stained with
32   PAS, a procedure designed to minimize glycogen loss. Kato-Weinstein et al. (2001) reported
33   that glycogen rich and poor cells were scattered without zonal distribution in male B6C3F1 mice
34   exposed to 2 g/l DCA for 8 weeks. For TCA treatments they reported centrilobular decreases in
35   glycogen and ~ 25% decreases in whole liver by 3 g/l TCA. Kato-Weinstein et al. (2001)
36   reported whole liver glycogen to be increased ~1.50-fold of control (90 vs. 60 mg glycogen/g
37   liver) by 2 g/l DCA after 8 weeks exposure male B6C3F1 mice with a maximal level of glycogen
38   accumulation occurring after 4 weeks of DCA exposure. Pereira et al. (2004) reported that after
39   8 weeks of exposure to 3.2 g/l DCA liver glycogen content was 2.20-fold of control levels (155.7
40   vs. 52.4. mg glycogen/g liver) in female B6C3F1 mice. Thus, the baseline level of glycogen
41   content reported by (~ 60 mg/g) and the increase in glycogen after DCA exposure was consistent
42   between Kato-Weinstein et al. (2001) and Pereira et al. (2004). However, the increase in liver
43   weight reported by Kato-Weinstein et al (2001) of 1.60-fold of control % liver/body weight

1 cannot be accounted for by the 1.50-fold of control glycogen content. Glycogen content only
2 accounts for 5% of liver mass so that 50% increase in glycogen cannot account for the 60%
3 increase liver mass induced by 2 g/l DCA exposure for 8 weeks reported by Kato-Weinstein
4 (2001). Thus, DCA-induced increases in liver weight are occurring from other processes as well.
5 Carter et al. (2003) and DeAngelo et al. (1999) reported increased glycogen after DCA treatment
6 at much lower doses after longer periods of exposure (100weeks). Carter reported increased
7 glycogen at 0.5 g/l DCA and DeAngelo et al (1999) at 0.03 g/l DCA in mice. However there is
8 no quantitation of that increase.
9
10 The issues involving identification of MOA through tumor phenotype analysis are
11 discussed in detail below for the more general case of liver cancer as well as for specific
12 hypothesized MOAs (see Sections 3.1.4., 3.1.8., 3.2.1., and 3.4.1.5). For TCE and its
13 metabolites, c-Jun staining, H-rats mutation, tincture, heterogeneity in dysplacity have been used
14 to describe and differentiate liver tumors in the mouse.
15
16 Bull et al. (2002) reported 1000 mg/kg TCE administered via gavage daily for 79 weeks
17 in male B6C3F1 mice to produce liver tumors and also reported deaths by gavage error (6 out of
18 40 animals). The limitations of the experiment are discussed in Caldwell et al (2007b)
19 Specifically, for the DCA and TCA exposed animals, the experiment was limited by low
20 statistical power, a relatively short duration of exposure, and uncertainty in reports of lesion
21 prevalence and multiplicity due to inappropriate lesions grouping (i.e., grouping of hyperplastic
22 nodules, adenomas, and carcinomas together as "tumors"), and incomplete histopatholology
23 determinations (i.e., random selection of gross lesions for histopathology examination). For the
24 TCE results, a high prevalence (23/36 B6C3F1 male mice) of adenomas and hepatocellular
25 carcinoma (7/36) was reported. For determinations of immunoreactivity to c-Jun, as a marker of
26 differences in "tumor" phenotype, Bull et al. (2002) included all lesions in most of their
27 treatment groups, decreasing the uncertainty of his findings. However, for immunoreactivity
28 results hyperplastic nodules, adenomas, and carcinomas were grouped and thus, changes in c-Jun
29 expression between the differing types of lesions were not determined. Bull et al. (2002)
30 reported lesion reactivity to c-Jun antibody to be dependent on the proportion of the DCA and
31 TCA administered after 52 weeks of exposure. Given alone, DCA was reported to produce
32 lesions in mouse liver for which approximately half displayed a diffuse immunoreactivity to a c-
33 Jun antibody, half did not, and none exhibited a mixture of the two. After TCA exposure alone,
34 no lesions were reported to be stained with this antibody. When given in various combinations,
35 DCA and TCA co-exposure induced a few lesions that were only c-Jun+, many that were only c-
36 Jun-, and a number with a mixed phenotype whose frequency increased with the dose of DCA.
37 For TCE exposure of 79 weeks, TCE-induced lesions were reported to also have a mixture of
38 phenotypes (42% c-Jun+, 34% c-Jun-, and 24% mixed) and to be most consistent with those
39 resulting from DCA and TCA co-exposure but not either metabolite alone.
40
41 Stauber and Bull (1997) exposed male B6C3F1 mice (7 weeks old at the start of
42 treatment) to 2.0 g/l neutralized DCA or TCA in drinking water for 38 or 50 weeks, respectively
43 and then exposed (n = 12) to 0, 0.02, 0.1, 0.5, 1.0, 2.0 g/L DCA or TCA for an additional 2

1 weeks. Foci and tumors were combined in reported results as "lesions" and prevalence rates
2 were not reported. The DCA-induced larger "lesions" were reported to be more "uniformly
3 reactive to c-Jun and c-Fos but many nuclei within the lesions displaying little reactivity to c-Jun.
4 Stauber and Bull (1997) stated that while most DCA-induced "lesions" were homogeneously
5 immunoreactive to c-Jun and C-Fos (28/41 lesions), the rest were stained heterogeneously. For
6 TCA-induced lesions, the authors reported no difference in staining between "lesions" and
7 normal hepatocytes in TCA-treated animals. These results are slightly different that those
8 reported by Bull et al. (2002) for DCA, who report c-Jun positive and negative foci in DCA-
9 induced liver tumors but no mixed lesions. Because "lesions" comprised of foci and tumors,
10 different stages of progression reported in these results. The duration of exposures also differed
11 between DCA and TCA treatment groups that can affect phenotype. The shorter duration of
12 exposure can also prevent full expression of the tumor response.
13
14   Stauber et al (1998) presented a comparison of *in vitro* results with "tumors" from
15 Stauber and Bull (1997) and note that 97.5% of DCA-induced "tumors" were c-Jun + while none
16 of the TCA-induced "tumors" were c-Jun +. However, the concentrations used to give tumors *in*
17 *vivo* for comparison with *in vitro* results were not reported. This appears to differ from the
18 heterogeneity of result for c-Jun staining reported by Bull et al (2002) and Stauber and Bull
19 (1997). There was no comparison of c-Jun phenotype for spontaneous tumors with the authors
20 stating that because of such short time, no control tumors results were given. However, the
21 results of Bull et al (2002) and Stauber and Bull (1997), do show TCA-induced lesions to be
22 uniformly c-Jun negative and thus the phenotypic marker was able to show that TCE-induced
23 tumors were more like those induced by DCA than TCA.
24
25   The premise that DCA induced c-Jun positive lesions and TCA-induced c-Jun negative
26 lesions in mouse liver was used as the rationale to study induction of "transformed" hepatocytes
27 by DCA and TCE treatment *in vitro*. Stauber et al. (1998) isolated primary hepatocytes from 5-8
28 week old male B6C3F1 mice (n=3) and subsequently cultured them in the presence of DCA or
29 TCA. In a separate experiment 0.5 g/l DCA was given to mice as pretreatment for 2 weeks prior
30 to isolation. The authors assumed that the anchorage-independent growth of these hepatocytes
31 was an indication of an "initiated cell." DCA and TCA solutions were neutralized before use.
32 After 10 days in culture with DCA or TCA (0, 0.2, 0.5 and 2.0 mM), concentrations of 0.5 mM
33 or more DCA and TCA both induced an increase in the number of colonies that was statistically
34 significant, increased with dose with DCA, and slightly greater for DCA. In a time course
35 experiment the number of colonies from DCA treatment *in vitro* peaked by 10 days and did not
36 change through days 15- 25 at the highest dose and, at lower concentrations of DCA, increased
37 time in culture induced similar peak levels of colony formation by days 20-25 as that reached by
38 10 days at the higher dose. Therefore, the number of colonies formed was independent of dose if
39 the cells were treated long enough *in vitro*. However, not only did treatment with DCA or TCA
40 induce anchorage independent growth but untreated hepatocytes also formed larger numbers of
41 colonies with time, although at a lower rate than those treated with DCA. The level reached by
42 untreated cells in tissue culture at 20 days was similar to the level induced by 10 days of
43 exposure to 0.5 mM DCA. The time course of TCA exposure was not tested to see if it had a

1 similar effect with time as did DCA. The colonies observed at 10 days were tested for c-Jun
2 expression with the authors noting that "colonies promoted by DCA were primarily c-Jun
3 positive in contrast to TCA promoted colonies that were predominantly c-Jun negative." Of the
4 colonies that arose spontaneously from tissue culture conditions, 10/13 (76.9%) were reported to
5 be c-Jun +, those treated with DCA 28/34 (82.3%) were c-Jun +, and those treated with TCA
6 5/22 (22.7%) were c-Jun +.   Thus, these data show heterogeneity in cell in colonies but with
7 more were c-Jun + colonies occurring by tissue culture conditions alone and in the presence of
8 DCA, rather than in the presence of TCA. The authors reported that with time (24, 48, 72, and
9 96 hours) of culture conditioning the number of c-Jun+ colonies was increased in untreated
10 controls.   The authors reported that DCA treatment delayed the increase in c-Jun+ expression
11 induced by tissue culture conditions alone in untreated controls while TCA treatment was
12 reported to not affect the increasing c-Jun+ expression that increased with time in tissue culture.
13 This results seems paradoxical given that DCA induced a higher number of colonies at 10 days
14 of tissue culture than TCA and that most of the colonies were c-Jun positive. The number of
15 colonies was greater for pretreatment with DCA, but the magnitude of difference over the control
16 level was the same after DCA treatment *in vitro* without and without pretreatment. As to the
17 relationship of c-Jun staining and peroxisome proliferators as a class, as pointed out by Caldwell
18 and Keshava (2006), although Bull et al. (2004) have suggested that the negative expression of c-
19 *jun* in TCA-induced tumors may be consistent with a characteristic phenotype shown in general
20 by peroxisome proliferators as a class, there is no supporting evidence of this.
21
22   An approach to determine the potential MOAs of DCA and TCA through examination of
23 the types of tumors each "induced" or "selected" was to examine H-ras activation (Ferreira-
24 Gonzalez et al., 1995; Anna et al., 1994; Bull et al., 2002; Nelson et al., 1990. This approach has
25 also been used to try to establish an H-ras activation pattern for "genotoxic" and "non-genotoxic"
26 liver carcinogens compounds and to make inferences concerning peroxisome proliferator-
27 induced liver tumors. However, as noted by Stanley et al. (1994), the genetic background of the
28 mice used and the dose of carcinogen may affect the number of activated H-ras containing
29 tumors which develop. In addition, the stage of progression of "lesions" (i.e., foci vs. adenomas
30 vs. carcinomas) also has been linked the observance of H-ras mutations.   Fox et al. (1990) note
31 that tumors induced by phenobarbital (0.05% drinking H_2O, 1 yr), chloroform (200 mg/kg corn
32 oil gavage, 2 times weekly for 1 year) or Ciprofibrate (0.0125% diet, 2 years) had a much lower
33 frequency of H-ras gene activation than those that arose spontaneously (2-year bioassays of
34 control animals) or induced with the "genotoxic" carcinogen benzidine-2 HCl (120 ppm,
35 drinking H_2O, 1 yr) in mice. In that study, the term "tumor" was not specifically defined but a
36 correlation between the incidence of H-ras gene activation and development of either a
37 hepatocellular adenoma or hepatocellular carcinoma was reported to be made with no
38 statistically significant difference between the frequency of H-ras gene activation in the
39 hepatocellular adenomas and carcinomas. Histopathological examination of the spontaneous
40 tumors, tumors induced with benzidine-2HCL, Phenobarbital, and chloroform was not reported
41 to reveal any significant changes in morphology or staining characteristics. Spontaneous tumors
42 were reported to have 64% point mutation in codon 61 (n= 50 tumors examined) with a similar
43 response for Benzidine of 59% (n= 22 tumors examined), whereas for Phenobarbital the

1   mutation rate was 7% (n= 15 tumors examined), chloroform 21% (n= 24 tumors examined) and
2   Ciprofibrate 21% (n= 39 tumors examined). The Ciprofibrate-induced tumors were reported to
3   be more eosinophilic as were the surrounding normal hepatocytes. Hegi et al. (1993) tested
4   Ciprofibrate-induced tumors in the NIH3T3 co-transfection-nude mouse tumorigenicity assay,
5   which the authors state is capable of detecting a variety of activated proto-oncogenes. The
6   tumors examined (Ciprofibrate-induced or spontaneously arising) were taken from the Fox et al.
7   study (1990), screened previously, and found to be negative for H-ras activation. With the
8   limited number of samples examined, Hegi et al concluded that ras proto-oncogene activation or
9   activation of other proto-oncogenes using the nude mouse assay were not frequent events in
10 Ciprofibrate-induced tumors and that spontaneous tumors were not promoted with it. Using the
11 more sensitive methods, the H-ras activation rate was reported to be raised from 21 to 31% for
12 Ciprofibrate-induced tumors and from 64 to 66% for spontaneous tumors. Stanley et al. (1994)
13 studied the effect of Methylclofenapate (MCP) (25 mg/kg for up to 2 years), a peroxisome
14 proliferator, in B6C3F1 (relatively sensitive) and C57BL/10J (relatively resistant) mice for H-ras
15 codon 61 point mutations in MCP-induced liver tumors (hepatocellular adenomas and
16 carcinomas). In the B6C3F1 mice the number of tumors with codon 61 mutations was 11/46 and
17 for C57BL/10J mice 4/31. Unlike the findings of Fox et al (1990), Stanley et al (1994) reported
18 an increase in the frequency of mutation in carcinomas, which was reported to be twice that of
19 adenomas in both strains of mice, indicating that stage of progression was related to the number
20 of mutations in those tumors, although most tumors induced by MCP did not have this mutation.
21
22   In terms of liver tumor phenotype, Anna et al. (1994) reported that the H-ras codon 61
23 mutation frequency was not statistically different in liver tumors from DCA and TCE-treated
24 mice from a highly variable number of tumors examined. In regard to mutation spectra in H-ras
25 oncogenes in control or spontaneous tumors, the patterns were slightly different but mostly
26 similar to that of DCA-induced tumors (0.5% in drinking water). From their concurrent controls
27 they reported that H-ras codon 61 mutations in 17% (n=6) of adenomas and 100% (n=5) of
28 carcinomas. For historical controls (published and unpublished) they reported mutations in 73%
29 (n=33) of adenomas and mutations in 70% (n=30) of carcinomas.   For tumors from TCE treated
30 animals they reported mutations in 35% (n=40) of adenomas and 69% (n=36) of carcinomas,
31 while for DCA treated animals they reported mutations in 54% (n=24) of adenomas and in 68%
32 (n=40) of carcinomas. Anna et al 1994) reported more mutations in TCE-induced carcinomas
33 than adenomas.
34
35   The study of Ferreira-Gonzalez (1995) in male B6C3 F1 mice has the advantage of
36 comparison of tumor phenotype at the same stage of progression (hepatocellular carcinoma), for
37 allowance of the full expression of a tumor response (i.e. 104 weeks), and an adequate number of
38 spontaneous control lesions for comparison with DCA or TCA treatments. However, tumor
39 phenotype at an endstage of tumor progression reflects of tumor progression and not earlier
40 stages of the disease process. In spontaneous liver carcinomas, 58% were reported to show
41 mutations in H-61 as compared with 50% of tumor from 3.5 g/l DCA-treated mice and 45% of
42 tumors from 4.5.g/l TCA-treated mice. Thus, there was a heterogeneous response for this
43 phenotypic marker for the spontaneous, DCA-, and TCA- treatment induced hepatocellular

1 carcinomas and not a pattern of reduced H-ras mutation reported for a number of peroxisome
2 proliferators. A number of peroxisome proliferators have been reported to have a much smaller
3 mutation frequency that spontaneous tumors [e.g,. 13-24% H-ras codon 61 mutations after
4 Methylclofenopate depending on mouser strain, Stanley et al. (1994): 21 to 31% for
5 Ciprofibrate-induced tumors and from 64 to 66% for spontaneous tumors, Fox et al. (1990) and
6 Hegi et al (1993)].
7
8   Bull (2000) suggested that "the report by Anna et al (1994) indicated that TCE-induced
9 tumors possessed a different mutation spectra in codon 61 of the H-ras oncogene than those
10 observed in spontaneous tumors of control mice." Bull (2000) stated that "results of this type
11 have been interpreted as suggesting that a chemical is acting by a mutagenic mechanism" but
12 went on to suggest that it is not possible to a priori rule out a role for selection in this process and
13 that differences in mutation frequency and spectra in this gene provide some insight into the
14 relative contribution of different metabolites to TCE-induced liver tumors. Bull (2000) noted
15 that data from Anna et al. (1994), Ferreira-Gonzalez et al (1995), and Maronpot et al (1995)
16 indicated that mutation frequency in DCA-induced tumors did not differ significantly from that
17 observed in spontaneous tumors. Bull (2000) also noted that the mutation spectra found in DCA-
18 induced tumors has a striking similarity to that observed in TCE-induced tumors, and DCA-
19 induced tumors were significantly different than that of TCA-induced liver tumors.
20
21   Bull et al. (2002) reported that mutation frequency spectra for the H-ras codon 61 in
22 mouse liver "tumors" induced by TCE (n= 37 tumors examined) were reported to be
23 significantly different than that for TCA (n= 41 tumors examined), with DCA-treated mice
24 tumors giving an intermediate result (n= 64 tumors examined). In this experiment, TCA-induced
25 "tumors" were reported to have more mutations in codon 61(44%) than those from TCE (21%)
26 and DCA (33%). This frequency of mutation in the H-ras codon 61 for TCA is the opposite
27 pattern as that observed for a number of peroxisome proliferators in which the number of
28 mutations at H-ras 61 in tumors has been reported to be much lower than spontaneously arising
29 tumors (see Section 3.4.1.5.).   Bull et al. (2002) noted that the mutation frequency for all TCE
30 ,TCA or DCA tumors was lower in this experiment than for spontaneous tumors reported in
31 other studies (they had too few spontaneous tumors to analyze in this study), but that this study
32 utilized lower doses and was of shorter duration than that of Ferreira-Gonzalez (1995). These
33 are additional concerns in addition to the effects of lesion grouping in which a lower stage of
34 progression is group with more advanced stages. In a limited subset of tumors that were both
35 sequenced and characterized histologically, only 8 of 34 (24%) TCE-induced adenomas but 9/15
36 (60%) of TCE-induced carcinomas were reported to have mutated H-ras at codon 61, which the
37 authors suggest is evidence that this mutation is a late event.
38
39   Thus, in terms of H-ras mutation, the phenotype of TCE-induced tumors appears to be
40 more like DCA-induced tumors (which are consistent with spontaneous tumors), or those
41 resulting from a co-exposure to both DCA and TCA (Bull et al., 2002), than from those induced
42 by TCA. As noted above, Bull et al. (2002) reported the mutation frequency spectra for the H-
43 ras codon 61 in mouse liver tumors induced by TCE to be significantly different than that for

1 TCA, with DCA-treated mice tumors giving an intermediate result and for TCA-induced tumors
2 to have a H-ras profile that is the opposite than those of a number of other peroxisome
3 proliferators. More importantly, these data suggest that using measures, other than dysplasticity
4 and tincture, mouse liver tumors induced by TCE are heterogeneous in phenotype.
5
6 　　　With regard to tincture, Stauber and Bull (1997) reported the for male B6C3F1 mice,
7 DCA-induced "lesions" contained a number of smaller lesions that were heterogeneous and more
8 eosinophilic with larger "lesions" tending to less numerous and more basophilic. For TCA
9 results using this paradigm, the "lesions" were reported to be less numerous, more basophilic,
10 and larger than those induced by DCA. Carter et al. (2003) used tissues from the DeAngelo et al.
11 (1999) and examined the heterogeneity of the DCA-induced lesions and the type and phenotype
12 of preneoplastic and neoplastic lesions pooled across all time points. Carter et al. (2003)
13 examined the phenotype of liver tumors induced by DCA in male B6C3 F1 mice and the shape
14 of the dose-response curve for insight into its MOA. They reported a dose-response of
15 histopathologic changes (all classes of premalignant lesions and carcinomas) occurring in the
16 livers of mice from 0.05–3.5 g/L DCA for 26–100 weeks and suggest foci and adenomas
17 demonstrated neoplastic progression with time at lower doses than observed DCA genotoxicity.
18 Preneoplastic lesions were identified as eosinophilic, basophilic and/or clear cell (grouped with
19 clear cell and mixed cell) and dysplastic. Altered foci were 50% eosinophilic with about 30%
20 basophilic. As foci became larger and evolved into carcinomas they became increasingly
21 basophilic. The pattern held true through out the exposure range. There was also a dose and
22 length of exposure related increase in atypical nuclei in "non-involved" liver. Glycogen
23 deposition was also reported to be dose-dependent with periportal accumulation at the 0.5 g/l
24 exposure level. Carter et al. (2003) suggested that size and evolution into a more malignant state
25 are associated with increasing basophilia, a conclusion consistent with those of Bannasch (1996)
26 and that there a greater periportal location of lesions suggestive as the location from which they
27 arose. Consistent with the results of DeAngelo et al (1999), Carter et al. (2003) reported that
28 DCA (0.05 – 3.5 g/l) increased the number of lesions per animal relative to animals receiving
29 distilled water, shortened the time to development of all classes of hepatic lesions, and that the
30 phenotype of the lesions were similar to those spontaneously arising in controls. Along with
31 basophilic and eosinophilic lesions or foci, Carter et al (2003) concluded that DCA-induced
32 tumors also arose from isolated, highly dysplastic hepatocytes in male B6C3F1 mice chronically
33 exposed to DCA suggesting another direct neoplastic conversion pathway other than through
34 eosinophilic or basophilic foci.
35
36 　　　Rather than male B6C3F1 mice, Pereira (1996) studied the dose-response relationship for
37 the carcinogenic activity of DCA and TCA and characterized their lesions (foci, adenomas and
38 carcinomas) by tincture in females (the generally less sensitive gender). Like the studies of TCE
39 by Maltoni et al (1986), female mice were also reported to have increased liver tumors after TCA
40 and DCA exposures. Pereira (1996) pool lesions were pooled for phenotype analysis so the
41 affect of duration of exposure could not be determined nor adenomas separated from carcinomas
42 for "tumors."   However, as the concentration of DCA was decreased the number of foci was
43 reported by Pereira (1996) to be decreased but the phenotype of the foci to go from primarily

1   eosinophilic foci (i.e. ~ 95% eosinophilic at 2.58 g/l DCA) to basophilic foci (~ 57%
2   eosinophilic at 0.26 g/l). For TCA the number of foci was reported to ~ 40 basophilic and ~ 60
3   eosinophilic regardless of dose. Spontaneously occurring foci were more basophilic by a ratio of
4   7/3. Pereira (1996) described the foci of altered hepatocytes and tumors induced by DCA in
5   female B6C3F1 mice to be eosinophilic at higher exposure levels but at lower or intermittent
6   exposures to be half eosinophilic and half basophilic. Regardless of exposure level, half of the
7   TCA-induced foci were reported to be half eosinophilic and half basophilic with tumors 75%
8   basophilic. In control female mice, the limited numbers of lesions were mostly basophilic, with
9   most of the rest being eosinophilic with the exception of a few mixed tumors. The limitations of
10 descriptions tincture and especially for inferences regarding peroxisome proliferator from the
11 description of "basophilia" is discussed in Section 3.4.1.5.
12
13   The results appear to differ between male and female B6C3F1 mice in regard to tincture
14 for DCA and TCA at differing doses. What is apparent is that the tincture of the lesions is
15 dependent on the stage of tumor progression, agent (DCA or TCA), gender, and dose. Also what
16 is apparent from these studies is the both DCA and TCA are heterogeneous in their tinctoral
17 characteristics as well as phenotypic markers such as mutation spectra or expression of c-Jun.
18
19   The descriptions of tumors in mice reported by the NCI, NTP, and Maltoni et al studies
20 are also consistent with phenotipic heterogeneity as well as consistency with spontaneous tumor
21 morphology (see Section 3.4.1.5). As noted in section 3.1, hepatocellular carcinomas observed
22 in humans are also heterogeneous. For mice, Maltoni et al (1986) described malignant tumors of
23 hepatic cells to be of different sub-histotypes, and of various degrees of malignancy and were
24 reported to be unique or multiple, and have different sizes (usually detected grossly at necropsy)
25 from TCE exposure. In regard to phenotype tumors were described as usual type observed in
26 Swiss and B6C3F1 mice, as well as in other mouse strains, either untreated or treated with
27 hepatocarcinogens and to frequently have medullary (solid), trabecular, and pleomorphic
28 (usually anaplastic) patterns. For the NCI (1976) study, the mouse liver tumors were described
29 in detail and to be heterogeneous "as described in the literature" and similar in appearance to
30 tumors generated by carbon tetrachloride. The description of liver tumors in this study and
31 tendency to metastasize to the lung are similar to descriptions provided by Maltoni et al (1986)
32 for TCE-induced liver tumors in mice via inhalation exposure. The NTP (1990) study reported
33 TCE exposure to be associated with increased incidence of hepatocellular carcinoma (tumors
34 with markedly abnormal cytology and architecture) in male and female mice. Hepatocellular
35 adenomas were described as circumscribed areas of distinctive hepatic parenchymal cells with a
36 perimeter of normal appearing parenchyma in which there were areas that appeared to be
37 undergoing compression from expansion of the tumor. Mitotic figures were sparse or absent but
38 the tumors lacked typical lobular organization. Hepatocellular carcinomas were reported to have
39 markedly abnormal cytology and architecture with abnormalities in cytology cited as including
40 increased cell size, decreased cell size, cytoplasmic eosinophilia, cytoplasmic basophilia,
41 cytoplasmic vacuolization, cytoplasmic hyaline bodies and variations in nuclear appearance.
42 Furthermore, in many instance several or all of the abnormalities were reported to be present in
43 different areas of the tumor and variations in architecture with some of the hepatocellular

1   carcinomas having areas of trabecular organization. Mitosis was variable in amount and
2   location. Therefore the phenotype of tumors reported from TCE exposure was heterogenous in
3   appearance between and within tumors from all 3 of these studies.
4
5   Caldwell and Keshava (2006) report "that Bannasch (2001) and Bannasch et al. (2001)
6   describe the early phenotypes of preneoplastic foci induced by many oncogenic agents (DNA-
7   reactive chemicals, radiation, viruses, transgenic oncogenes and local hyperinsulinism) as
8   insulinomimetic. These foci and tumors have been described by tincture as eosinophilic and
9   basophilic and to be heterogeneous. The tumors derived from them after TCE exposure are
10   consistent with the description for the main tumor lines of development described by Bannasch
11   et al (2001) (see Section 3.4.1.5). Thus, the response of liver to DCA (glycogenosis with
12   emergence of glycogen poor tumors) is similar to the progression of preneoplastic foci to tumors
13   induced from a variety of agents and conditions associated with increased cancer risk."
14   Furthermore Caldwell and Keshava (2006) note that Bull et al. (2002) report expression of IR to
15   be elevated in tumors of control mice or mice treated with TCE, TCA and DCA but not in non-
16   tumor areas suggesting that this effect is not specific to DCA.
17
18
19   There is a body of literature that has focused on the effects of TCE and its metabolites
20   after rats or mice have been exposed to "mutagenic" agents to "initiate" hepatocarcinogenesis
21   and this is discussed in Section 4.2 below. TCE and its metabolites were reported to affect tumor
22   incidence, multiplicity, and phenotype when given to mice as a co-exposure with a variety of
23   "initiating" agents and with other carcinogens. Pereira and Phelps (1996) reported that MNU
24   alone induced basophilic foci and adenomas. MNU and low concentrations of DCA or TCA in
25   female mice were reported to induce heterogeneous for foci and tumor with a higher
26   concentration of DCA inducing more eosinophilic and a higher concentration of TCA inducing
27   more tumors that were basophilic. Pereira et al (2001) reported that not only dose, but gender
28   also affected phenotype in mice that had already been exposed to MNU and were then exposed
29   to DCA. As for other phenotypic markers, Lantendresse and Pereira (1997) reported that
30   exposure to MNU and TCA or DCA induced tumors that had some commonalities, were
31   heterogeneous, but for female mice were overall different between DCA and TCA as co-
32   exposures with MNU.
33
34   Stop experiments which attempt to ascertain the whether progression differences exist
35   between TCA and DCA have used higher concentrations at much lower durations of exposure.
36   A question arises as to whether the differences in results between those animals in which
37   treatment was suspended in comparison to those in which had not had been conducted so that full
38   expression of response had not been allowed rather than "progression" as well as the effects of
39   using large doses. After 37 weeks of treatment and then a cessation of exposure for 15 weeks
40   Bull et al. (1990) reported that after 15 weeks of cessation of exposure, liver weight and %
41   liver/body weight were reported to still be statistically significantly elevated after DCA or TCA
42   treatment. The authors partially attribute the remaining increases in liver weight to the continued
43   presence of hyperplastic nodules in the liver. In terms of liver tumor induction, the authors

1 stated that "statistical analysis of tumor incidence employed a general linear model ANOVA
2 with contrasts for linearity and deviations from linearity to determine if results from groups in
3 which treatments were discontinued after 37 weeks were lower than would have been predicted
4 by the total dose consumed." The multiplicity of tumors observed in male mice exposed to DCA
5 or TCA at 37 weeks and then sacrificed at 52 weeks were reported by the authors to have a
6 response in animals that received DCA very close to that which would be predicted from the
7 total dose consumed by these animals. The response to TCA was reported by the authors to
8 deviate significantly (P= 0.022) from the linear model predicted by the total dose consumed.
9 Multiplicity of lesions per mouse and not incidence was used as the measure. Most importantly
10 the data used to predict the dose response for "lesions" used a different methodology at 52 weeks
11 than those at 37 weeks. Not only were not all animal's lesions examined, but foci, adenomas,
12 and carcinomas were combined into one measure. Therefore foci, of which a certain percentage
13 have been commonly shown to spontaneously regress with time, were included in the calculation
14 of total "lesions". Pereira and Phelps (1996) note that in MNU-treated mice that were then
15 treated with DCA, the yield of altered hepatocytes decreases as the tumor yields increase
16 between 31 and 51 weeks of exposure suggesting progression of foci to adenomas. Initiated and
17 non-initiated control mice were reported to also have fewer foci/mouse with time. Because of
18 differences in methodology and the lack of discernment between foci, adenomas, and carcinomas
19 for many of the mice exposed for 52 weeks, it is difficult to compare differences in composition
20 of the "lesions" after cessation of exposure in the Bull et al (1990) study. For TCA treatment the
21 number of animals examined for determination of which "lesions" were foci, adenomas, and
22 carcinomas was 11 out of the 19 mice with "lesions" at 52 weeks while all 4 mice with lesions
23 after 37 weeks of exposure and 15 weeks of cessation were examined. For DCA treatment the
24 number of animals examined was only 10 out of 23 mice with "lesions" at 52 weeks while all 7
25 mice with lesions after 37 weeks of exposure and 15 weeks of cessation were examined. Most
26 importantly, when lesions were examined microscopically then did not all turn out to be
27 preneoplastic or neoplastic. Two lesions appeared "to be histologically normal" and one
28 necrotic. Not only were a smaller number of animals examined for the cessation exposure than
29 continuous exposure but only the 2 g/l exposure levels of DCA and TCA were studied for
30 cessation. The number of animals bearing "lesions" at 37 and then 15 week cessation weeks was
31 7/11 (64%) while the number of animals bearing lesions at 52 weeks was 23/24 (96%) after 2 g/l
32 DCA exposure. For TCA the number of animals bearing lesions at 37 weeks and then 15 weeks
33 cessation was 4/11 (35%) while the number of animals bearing lesions at 52 weeks was 19/24
34 (80%). While suggesting that cessation of exposure diminished the number of "lesions,"
35 conclusions regarding the identity and progression of those lesion with continuous vs. non-
36 continuous DCA and TCA treatment are tenuous.
37
38 ## 2.5. Studies of Chloral Hydrate
39
40 Given that total oxidative metabolism appears to be highly correlated with TCE-induced
41 increases in liver weight in the mouse rather than merely the presence of TCA, other metabolites
42 are of interest as potential agents mediating the effects observed for TCE. Recently Caldwell
43 and Keshava provided a synopsis of the results of more recent studies involving chloral hydrate

1   (CH) (Caldwell and Keshava, 2006). A large fraction of TCE oxidative metabolism appears to
2   go through chloral hydrate, with subsequent metabolism to TCA and trichloroethanol (Chiu et
3   al., 2006b). Merdink et al. (2008) demonstrated that CH administered to humans can be
4   extremely variable and complex in its pharmacokinetic behaviour with a peak plasma
5   concentration of CH in plasma 40-50 times higher than observed at the same time interval for
6   other subjects. Studies of CH toxicity in rodents are consistent with the general presumption that
7   oxidative metabolites are important for TCE-induced liver tumors, but whether CH and its
8   metabolites are sufficient to explain all of TCE liver tumorigenesis remains unclear, particularly
9   because of uncertainties regarding how DCA may be formed (Chiu et al., 2006b). Studies of CH
10   may enable a comparison between toxicity of TCE and CH and may help elucidate its role in
11   TCE effects. As with other TCE metabolites, the majority of the studies have focused on the
12   mouse liver tumor response. For rats, while the limited data suggests that there is less of a
13   response than mice to CH, those studies are limited in power or reporting.
14
15   Daniel et al. (1992) exposed adult male B6C3F1 (C57B1/6jC male mice bred to
16   C3Heb/Fej female mice) 28-day old mice to CH, 2-chloroacetaldehyde, or DCA in 2 different
17   phases (I and II) with initial weights ranging from 9.4 to 13.6 g. The test compounds were
18   buffered and administered in drinking water for 30 and 60 weeks (n=5 for interim sacrifice), and
19   for 104 weeks (n=40). The concentration of CH was 1 g/l and for DCA 0.5 g/l and the estimated
20   doses of DCA were 85 mg/kg/day, 93 mg/kg/day and 166 mg/kg/day for the DCA group I, DCA
21   group II, and CH exposed group, respectively. Microscopic examination of tissues was
22   conducted for all tissues for five animals of the CH groups with liver, kidneys, testes, and spleen,
23   in addition to all gross lesions, reported to be examined microscopically in all of the 104-week
24   survivors. The initial body weight for drinking water controls was reported to be 12.99 ± 3.04 g
25   for group I (n= 23) and 10.48 ± 1.70 for group II (n =10).   For DCA treated animals, initial
26   body weights were 13.44 ± 2.57 g for group I (n=23) and 9.65 ± 2.72 g for group II (n= 10). For
27   the CH treated group the initial body weights were reported to be 10.42 ± 2.49 g (n= 40). It is
28   not clear from the report what control group best matched, if any, the CH group. Thus, the mean
29   initial body weights of the groups as well as the number of animals varied considerably in each
30   group (i.e., ~ 40% difference in mean body weights at the beginning of the study). The number
31   of animals surviving till the termination of the experiment was 10, 10, 16, 8, and 24 for the
32   control group I, control group II, DCA group I, DCA group II, and CH groups, respectively. An
33   increase in absolute and relative liver weight vs. reported to be observed at 30 weeks for DCA
34   and CH groups and at 60 weeks for CH but data was not shown in the study. At 104 weeks, the
35   data for the surviving control groups were combined as was that for the 2 DCA treatment groups.
36   Of note was that for CH treated survivors (n=24) water consumption was significantly reduced in
37   comparison to controls. Absolute liver weight was reported to be 2.09 ± 0.6 g, 3.17 ± 1.3 g and
38   2.87 ± 1.1. g for control, DCA and CH treatment groups, respectively. The % liver to body
39   weight was reported to be similarly elevated (1.57-fold of control for DCA and 1.41-fold of
40   control for CH) at 104 weeks. At 104 weeks the treatment-related liver lesions in histological
41   sections were reported to be most prominently hepatocytomegaly and vacuolization in DCA-
42   treated animals. Cytomegaly was also reported to be in 5%, 92%, and 79 % of control, DCA and
43   CH treatment groups, respectively. Cytomegaly in CH treated mice was described as minimal

1   and associated with an increased number of basophilic granules (rough endoplasmic reticulum).
2   Hepatocellular necrosis and chronic active inflammation were reported to be mildly increased in
3   both prevalence and severity in all treated groups. The histological findings, from interim
4   sacrifices (n=5), were considered by the authors to be unremarkable and were not reported.
5   Liver tumors were increased by DCA and CH treatment. The % incidence of liver carcinomas
6   and adenomas combined in the surviving animals was 15%, 75%, and 71% in control, DCA and
7   CH treated mice, respectively. In the CH treated group, the incidence of hepatocellular
8   carcinoma was 46%. The number of tumors/animals was also significantly increased with CH
9   treatment. Most importantly, morphologically the authors noted that there did not appear to be
10 any discernable differences in the visual appearance of the DCA- and CH-induced tumors.
11
12   George et al. (2000) exposed male B6C3F1 mice and male F344/N rats to CH in drinking
13 water for 2 years (up to 162.6 mg/kg/day). Target drinking water concentrations were 0, 0.05,
14 0.5, and 2 g/l CH in rats and 0, 0.05, 0.5 and 1.0 g/l CH in mice. Groups of animals (n=6/group)
15 were sacrificed at 13 (rats only), 26, 52 and 78 weeks following the initiation of dosing with
16 terminal sacrifices at week 104.   A complete pathological examination was performed on 5 rats
17 and mice from the high-dose group, with examination primarily of gross lesions except for liver,
18 kidney, spleen and testes. BrdU incorporation was measured in the interim sacrifice groups in
19 rats and mice with PCO examined at 26 weeks in mice.   In rats, the number of animals surviving
20 > 78 weeks and examined for hepatocellular proliferative lesions was 42, 44, 44, and 42 for the
21 control, 7.4, 37.4 and 163.6 mg/kg/day CH treatment groups, respectively. Only 32, 36, 35, and
22 32 animals were examined at the final sacrifice time. Only the lowest treatment group had
23 increased liver tumors which were marginally significantly increased by treatment. The % of
24 animals with hepatocellular adenomas and carcinomas was reported to be 2.4, 14.3, 2.3 and 6.8
25 % in male rats. In mice, preneoplastic foci and adenomas were reported to be increased in the
26 livers of all CH treatment groups (13.5–146.6 mg/kg/day) at 104 weeks. The incidences of
27 adenomas were reported to be statistically increased at all dose levels, the incidences of
28 carcinomas significantly increased at the highest dose, and time-to-tumor decreased in all CH-
29 treatment groups. The % incidence of hepatocellular adenomas was reported to be 21.4%,
30 43.5%, 51.3%, and 50% in control, 13.5, 65.0 and 146.6 mg/kg day treatment groups,
31 respectively. The % incidence of hepatocellular carcinomas was reported to be 54.8%, 54.3%,
32 59.0% and 84.4% in these same groups. The resulting % incidence of hepatocellular adenomas
33 and carcinomas was reported to be 64.3%, 78.3%, 79.5% and 90.6%. The number of mice
34 surviving > 78 weeks was reported to be 42, 46, 39, and 32 and the number surviving to final
35 sacrifice to be 34, 42, 31, and 25 for control, 13.5, 65.0 and 146.56 mg/kg/day, respectively. CH
36 exposure was reported to not alter serum chemistry, hepatocyte proliferation (i.e., DNA
37 synthesis), or hepatic PCO activity (an enzyme associated with PPARα agonism) in rats and
38 mice at any of the time periods monitored (all interim sacrifice periods for BrdU incorporation,
39 52 or 78 weeks for serum enzymes, and 26 weeks for PCO) with the exception of 0.58 g/L
40 chloral hydrate at 26 weeks slightly increasing hepatocyte labeling (~ 2-3 fold increase over
41 controls) in rats and mice but the % labeling still represented 3% or less of hepatocytes. With
42 regard to other carcinogenic endpoints only five animals were examined at the high dose, thereby
43 limiting the study's power to determine an effect. Control mice were reported to have a high

1 spontaneous carcinoma rate (54%), thereby limiting the ability to detect a treatment-related
2 response. No descriptions of the foci or tumor phenotype were given. However, of note is the
3 lack of induction of PCO response with CH at 26 weeks of administration in either rats or mice.
4
5   Leakey et al. (2003a) studied the effects of CH exposure (0, 25, 50, and 100 mg/kg, 5
6 days/week, 104–105 weeks via gavage) in male B6C3F1 mice with dietary control used to
7 manipulate body growth (n= 48 for 2 year study and n= 12 for the 15-month interim study).
8 Dietary control was reported to decrease background liver tumor rates (incidence of 15–20%)
9 and was reported to be associated with decreased variation in liver-to-body weight ratios, thereby
10 potentially increasing assay sensitivity. In dietary-controlled groups and groups fed *ad libitum*,
11 liver adenomas and carcinomas (combined) were reported to be increased with CH treatment.
12 With dietary restriction there was a more discernable CH tumor-response with overall tumor
13 incidence reduced, and time-to-tumor increased by dietary control in comparison to ad libitum
14 fed mice. Incidences of hepatocellular adenoma and carcinoma overall rates were reported to be
15 33%, 52%, 49%, and 46% for control, 25mg/kg, 50 mg/kg, and 100 mg/kg ad libitum-fed mice,
16 respectively. For dietary controlled mice the incidence rates were reported to be 22.9%, 22.9%,
17 29.2% and 37.5% for controls, 25mg/kg, 50 mg/kg, and 100 mg/kg CH, respectively. Body
18 weights were matched and carefully controlled in this study.
19
20   After 2 years of CH treatment the heart weights of ad libitum-fed male mice administered
21 100 mg/kg CH were reported to be significantly less and kidney weights of the 50 and 100
22 mg/kg less than vehicle controls. No other significant organ weight changes due to CH
23 treatment were reported to be observed in either diet group except for liver. The liver weights of
24 CH treated groups for by dietary groups were reported to be increased at 2 years and the absolute
25 liver weights of dosed groups to be generally increased at 15 months with % liver/body weight
26 ratios increased in CH treated dietary-controlled mice at 15 months. There was 1.0-fold, 0.87-
27 fold, and 1.08-fold of control % liver/body weight for ad libitum fed mice exposed to 25, 50 and
28 100 mg/kg CH, respectively. For dietary controlled mice, there was 1.05-fold, 1.08-fold, and
29 1.11-fold of control % liver/body weight for the same dose groups at 15 months. Thus, there was
30 no corresponding dose-response for % liver/body weight in the ad libitum-fed mice, which were
31 reported to show a much larger variation in liver-to-body- weight ratios (i.e., the standard
32 deviation and standard errors were 2- to 17-fold lower in dietary controlled groups than for ad
33 libitum-fed groups). Liver weight increases at 15-months did not correlate with 2-year tumor
34 incidences with this group. However for dietary controlled groups the increase in % liver/body
35 weights at 15 months were generally correlated with increases in liver tumors at 2 years. The
36 incidences of peripheral or focal fatty change were reported to be increased in all CH-treated
37 groups of ad libitum-fed mice at 15 months (~ half the animals showed these changes for all dose
38 groups, with no apparent dose-response). Of the enzymes associated with PPARα agonism (total
39 CYP, CYP2B isoform, CYP4A, or lauric acid β-hydroxylase activity), only CYP4A and lauric
40 acid β-hydroxylase activity were significantly increased at 15 months of exposure in the dietary-
41 restricted group administered 100 mg/kg CH with no other groups reported showing a
42 statistically significant increased response (n= 12/group). Although not statistically significant,
43 the 100 mg/kg CH exposure group of ad libitum-fed mice also had an increase in CYP4A and

1 lauric acid β-hydroxylase activity.   The authors reported that the increase in magnitude of
2 CYP4A and lauric acid β-hydroxylase activity at 100 mg/kg CH at 15 months in dietary
3 controlled mice correlated with the increase incidence of mice with tumors. However, there was
4 no correlation of tumor incidence and the increased enzyme activity associated with peroxisome
5 proliferation in the ad libitum-fed mice. No descriptions of liver pathology were given other
6 than incidence of mice with fatty liver changes. Hepatic malondialdehyde concentration in ad
7 libitum fed and dietary controlled mice did not change with CH exposure at 15 months but the
8 dietary controlled groups were all ~ half that of the ad libitum-fed mice. Thus, while overall
9 increased tumors observed in the ad libitum diet correlated with increased malondialdehyde
10 concentration, there was no association between CH dose and malondialdehyde induction for
11 either diet.
12
13   Induction of peroxisome-associated enzyme activities was also reported for shorter times
14 of CH exposure. Seng et al. (2003) described CH toxicokinetics in mice at doses up to 1,000
15 mg/kg/day for 2 weeks with dietary control and caloric restriction slightly reducing acute
16 toxicity. Lauric acid β-hydroxylase and PCO activities were reported to be induced only at doses
17 > 100 mg/kg in all groups, with dietary-restricted mice showing the greatest induction.
18 Differences in serum levels of TCA, the major metabolite remaining 24 hr after dosing, were
19 reported not to correlate with hepatic lauric acid β-hydroxylase activities across groups.
20
21   Leuschner and Beuscher (1998) examined the carcinogenic effects of CH in male and
22 female Sprague-Dawley rats (69-79 g , 25-29 days old at initiation of the experiment)
23 administered 0, 15, 45, and 135 mg/kg CH in unbuffered drinking water 7 days/week (n=
24 50/group) for 124 weeks in males and 128 weeks in females. Two control groups were noted in
25 the methods section without explanation as to why they were conducted as two groups. The
26 mean survival for males was similar in treated and control groups with 20%, 24%, 20%, 24%,
27 and 20 % of control I, control II, 15, 45, and 135 mg/kg CH-treated groups, respectively,
28 surviving till the end of the study. For female rats, the % survival was 12%, 30%, 24%, 28%,
29 and 16% for of control I, control II, 15, 45, and 135 mg/kg CH-treated groups, respectively. The
30 authors report no substance-related influence on organ weights and no macroscopic evidence of
31 tumors or lesions in male or female rats treated with CH for 124 or 128 weeks. However, no
32 data is presented on the incidence of tumors using this paradigm, especially background rates.
33 The authors report a statistically significant increase in the incidence of hepatocellular
34 hypertrophy in male rats at the 135 mg/kg dose (14/50 animals vs. 4/50 and 7/50 in controls I
35 and II). For female rats, the incidence of hepatocellular hypertrophy was reported to be 10/50
36 rats (control I) and 16/50 (control II) rats with 18/50, 13/50 and 12/50 female rats having
37 hepatocellular hypertrophy after 15, 45, and 135 mg/kg CH, respectively. The lack or reporting
38 in regard to final body weights, histology, and especially background and treatment group data
39 for tumor incidences, limit the interpretation of this study. Whether this paradigm was sensitive
40 for induction of liver cancer cannot be determined.
41
42   From the CH studies in mice, there is an apparent increase in liver adenomas and
43 carcinomas induced by CH treatment by either drinking water or gavage with all available

1 studies performed in male B6C3F1 mice. However, the background levels of hepatocellular
2 adenomas and carcinomas in these mice in George et al (2000) and body weight data from this
3 study show it is from a tumor prone mouse. Comparisons with concurrent studies of mice
4 exposed to DCA revealed that while both CH and DCA induced hepatomegaly and cytomegaly,
5 DCA-induced cytomegaly was accompanied by vacuolization while that of CH to be associated
6 with increased number of basophilic granules (rough endoplasmic reticulum) which would
7 suggest separate effects. However, the morphology of the CH-induced tumors was reported to
8 be similar between DCA and CH-induced tumors (Daniels et al., 1992). Using a similar
9 paradigm (2-year study of B6C3F1 male mice), De Angelo et al. (1999) and Carter et al. (2003)
10 described DCA-induced tumors to be heterogeneous. This is the same description given for
11 TCE-induced tumors in the studies by NTP, NCI, and Maltoni et al. and to be a common
12 description for tumors caused by a variety of carcinogenic agents. Similar to the studies cited
13 above for CH, DeAngelo et al (1999) reported that PCO levels were only elevated at 26 weeks at
14 3.5 g/l DCA and had returned to control levels by 52 weeks. Similar to CH, no increased
15 tritiated thymidine was reported for DCA at 26 and 52 weeks with only 2-fold of control values
16 reported at 0.05 g/l at 4 weeks. Leakey et al. (2003a) reported that ad libitum fed male mice
17 exhibited a similar degree of increased incidence of peripheral or focal fatty change at 15 months
18 for all CH doses but not enzymes associated with peroxisome proliferation. While dietary
19 restriction seemed to have decreased background levels of tumors and increased time-to-tumor,
20 CH-gave a clear dose-response in dietary restricted animals. However, while the overall level of
21 tumor induction was reduced there was a greater induction of PPARα enzymes by CH.
22 Induction of liver tumors by CH observed in ad libitum fed mice were not correlated with
23 PPARα induction, with dietary restriction alone appearing to have greater levels of lauric acid ω-
24 hydrolase activity in control mice at 15 months. Seng et al. (2003) report that lauric acid β-
25 hydroxylase and PCO were induced only at exposure levels > 100 mg/kg CH, again with dietary
26 restricted groups showing the greatest induction. Such data argues against the role of
27 peroxisome proliferation in CH-liver tumor induction in mice.
28
29 **2.6. Serum Bile Acid Assays**
30
31 Serum bile acids (SBA) have been suggested as a sensitive indicator of hepatotoxicity to
32 a variety of halogenated solvents with an advantage of increased sensitivity and specificity over
33 conventional liver enzyme tests that primarily reflect the acute perturbation of hepatocyte
34 membrane integrity and "cell leakage" rather than liver functional capacity (i.e., uptake,
35 metabolism, storage, and excretion functions of the liver) (Bai et al., 1992b; Neghab et al., 1997).
36 While some studies have reported negative results, a number of studies have reported elevated
37 SBA in organic solvent-exposed workers in the absence of any alterations in normal liver
38 function tests. These variations in results have been suggested to arise from failure of some
39 methods to detect some of the more significantly elevated SBA and the short-lived and reversible
40 nature of the effect (Neghab et al., 1997). Neghab et al. (1997) have reported that occupational
41 exposure to 1,1,2-trichloro-1,2,2-trifluoroethane and trichloroethylene has resulted in elevated
42 SBA and that several studies have reported elevated SBA in experimental animals to chlorinated
43 solvents such as carbon tetrachloride, chloroform, hexachlorobutadiene, tetrachloroethylene, 1,1,

1   1-trichloroethane, and trichloroethylene at levels that do not induce hepatotoxicity (Bai et al.
2   1992a, b; Hamdan and Stacey 1993,; Wang and Stacey 1990). Toluene, a non-halogenated
3   solvent, has also been reported to increase SBA in the absence of changes in other hepatobiliary
4   functions (Neghab and Stacey, 1997). Thus, disturbance in SAB appears to be a generalized
5   effect of exposure to chlorinated solvents and non-chlorinated solvents and not specific to TCE
6   exposure.
7
8   Neghab et al. (1997) reported that 8 hour time-weighted averages exposure to TCE of 8.9
9   ppm, measured in the breathing zone using a charcoal tube personal sampler for the whole mean
10   duration of exposure of 3.4 years, to have not significant changes in albumin, bilirubin, alkaline
11   phosphatase, alanine aminotransferase, 5'-nucleosidase, γ-glutamyltransferase, but to have
12   significantly increased total serum bile acids. Not only were total bile acids significantly
13   increased in these TCE-exposed workers compared to controls (~ 2-fold of control), but,
14   specifically, deoxycholic acid and subtotal of free bile acids were increased.   Neghab et al.
15   (1997) do not show the data, but also report that "despite the apparent overall low level of
16   exposure, there was a very good correlations (r = 0.94) between the degree of increase in serum
17   concentration of total bile acids and level of TCE". Neghab et al (1997) note that while a
18   sensitive indicator or exposure to such solvents in asymptomatic workers, there is no indication
19   that actual liver injury occurs in conjunction with SAB increases.
20
21   Wang and Stacey (1990) administered TCE in corn oil via i.p. injection to male Sprague-
22   Dawley rats (300-500 g) at concentrations of 0.01, 0.1, 1, 5, and 10 mmol/kg on 3 consecutive
23   days (n = 4, 5, or 6) with liver enzymes and SBA examined 4 hours after the last TCE treatment.
24   At these dose, there were not differences between treated and control animals in regard to
25   alkaline phosphatase and sorbitol dehydrogenase concentrations and an elevation of alanine
26   aminotransferase only at the highest dose. However, there was generally a reported dose-related
27   increase in cholic acid, chenodeoxycholic acid, deoxycholic acid, taurocholic acid,
28   tauroursodeoxycholic acid with cholic acid and taurochlolic acid increased at the lowest dose.
29   The authors report that "examination of liver sections under light microscopy yielded no
30   consistent effects that could be ascribed to trichloroethylene." In the same study a rats were also
31   exposed to TCE via inhalation (n = 4) at 200 ppm for 28 days, and 1000 ppm for 6 hours/day.
32   Using this paradigm, cholic acid and taurocholic acid were significantly elevated at the 200 ppm
33   level, (~ 10 –fold and ~ 5-fold of control, respectively) with very large standard errors of the
34   mean. At the 1000 ppm level (6 hours, day) cholic acid and taurocholic acid were elevated to ~
35   2-fold of control but neither was statistically significant. The large variability in responses
36   between rats and the low number of rats tested in this paradigm limit its ability to determine
37   quantitative differences between groups. Nevertheless, without the complications associated
38   with i.p. exposure (see Section 2.2.1 above), both inhalation exposure of TCE at a relative low
39   exposure level was also associated with increased SBA levels. The authors stated that "no
40   increases in alanine amino transferase levels were observed in the rats exposed to
41   trichloroethylene via inhalation." No histopathology results were reported for rats exposed via
42   inhalation. As stated by Wang and Stacey (1990), "intraperitoneal injection is not particularly
43   relevant to humans" which was the rationale given for the inhalation exposure experiments in the

1 study. They point out that intestinal interactions require consideration because a major
2 determinant of SBA is their absorption from the gut and intestinal flora may play a role in bile
3 acid metabolism. They also note that grooming done by the experimental rats would probably
4 give small exposure via ingestion of TCE as well. However, Wang and Stacey (1990) reported
5 consistent results in terms of TCE-induced changes in SBA at relatively low concentrations by
6 either inhalation or i.p. routes of exposure that were not associated with other measures of
7 toxicity.
8
9 Hamdan and Stacey (1993) administered TCE in corn oil (1 mmol/kg) in male Sprague
10 Dawley rats (300 – 400 g) and followed the time-course of SBA elevation, TCE concentration
11 and trichloroethanol in the blood at 2, 4, 8, and 16 hours after dosing (n= 4,5, or 6 per group).
12 Liver and blood concentration of TCE were reported to peak at 4 hours while those of
13 trichloroethanol peaked at 8 hours after dosing. TCE levels were not detectable by 16 hours in
14 either blood or liver while those of trichloroethanol were still elevated. Elevations of SBA were
15 reported to parallel those of TCE with cholic acid and taurochloate acid reported to show the
16 highest levels of bile acids. The dose given was based on that reported by Wang and Stacey
17 (1990) to give no hepatotoxicity but an increase in SBA. The authors state that liver injury
18 parameters were checked and found unaffected by TCE exposure but do not show the data.
19 Thus, it was TCE concentration and not that of its metabolite that was most closely related to
20 changes in SBA and after a single exposure, the effect was reversible. In an *in vitro* study by Bai
21 and Stacey (1993), TCE was studied in isolated rat hepatocytes with TCE reported to cause a
22 dose-related suppression of initial rates of cholic acid and taurocholic acid but with no significant
23 effects on enzyme leakage and intracellular calcium contents, further supporting a role for the
24 parent compound in this effect. The authors noted that the changes in SBA result from
25 interference with a physiological process rather "than an event associated with significant
26 pathological consequences."
27

28 ## State of Science of Liver Cancer MOAs

29
30 The experimental evidence in mice shows that TCE and its metabolites induce foci,
31 hepatocellular adenomas, and carcinomas that are heterogeneous in nature as indicated by
32 phenotypic differences in tincture, mutational markers, or gene expression markers. The tumors
33 induced by TCE are reflective of phenotypes that are either similar to those induced by mixtures
34 of DCA and TCA exposure, or more like those induced by DCA. These tumors have been
35 described to be similar also to those arising spontaneously in mice or from chemically induced
36 hepatocarcinogenesis and to arise from preneoplastic foci, and in the case of DCA, single
37 dysplastic hepatocytes as well as foci. Hepatocellular carcinoma (HCC) observed in humans
38 also has been described to be heterogeneous and to be associated with formation of preneoplastic
39 nodules. Although several conditions have been associated with increased risk of liver cancer in
40 humans, the mechanism of HCC is unknown at this time. A great deal of attention has been
41 focused on predicting which cellular targets (e.g., "stem-cell" or mature hepatocyte) are
42 associated with HCC as well as on phenotypic markers in HCC that can provide insight not only
43 into MOA and origin of tumor, but also for prediction of clinical course. Examination of

1   pathways and epigenetic changes associated with cancer, and the relationship of these changes to
2   liver cancer are also discussed below. The field of cancer research has been transformed by the
3   recent discoveries of epigenetic changes and their role in cancer and chronic disease states. The
4   following discussion describes these advances but also the issues involved with the technologies
5   that have emerged to describe them (see Section 3.1.2., below). Exposure to TCE and its
6   metabolites, like many others, induces a heterogeneous response, even in a relatively
7   homogeneous genetic paradigm as the experimental laboratory rodent model. The importance of
8   phenotypic anchoring is a major issue in the study of any MOAs using these new technologies of
9   gene expression pattern. Although a large amount of information is now available using
10   microarray technologies and transgenic mouse models, specifically for TCE and in study of
11   suggested MOAs for TCE and its metabolites, use of these approaches has limitations that need
12   to be considered in the interpretation of data and conclusions derived from such data, especially
13   quantitative conclusions.
14
15   For TCE and its metabolites, the extent of acute to subchronic induction of hepatomegaly
16   correlated with hepatocellular carcinogenicity, although each had differing factors contributing
17   to that hepatomegaly from periportal glycogen deposition to hepatocellular hypertrophy and
18   increased polyploidy. The extent of transient DNA synthesis, peroxisome proliferation, or
19   cytotoxicity was not correlated with carcinogenicity. Hepatomegaly is also a predictor of
20   carcinogenicity for a number of other compounds in mice and rats. Allen et al. (2004) examined
21   the NTP database (87 compounds for rat and 83 for mice) and tried to correlate specific
22   hepatocellular pathology in prechronic studies with carcinogenic endpoints in the chronic 2-year
23   assays. The best single predictor of liver cancer in mice was hepatocellular hypertrophy.
24   Hepatocellular cytomegaly and hepatocyte necrosis also contributed, although the numbers of
25   positive findings were less than hypertrophy. With regard to genotoxicity studies, there was no
26   evidence of a correlation between mouse liver tumor chemicals and Salmonella or micronucleus
27   assay outcome. None of the prechronic liver lesions examined were correlated with either
28   Salmonella or Micronucleus assays. In rats no single prechronic liver lesions (when considered
29   individually) was a strong predictor of liver cancer in rats. The most predictive lesions was
30   hepatocellular hypertrophy. There was not significant correlation between liver tumors/toxicity
31   and the 2 mutagenicity measures. Although the lack of correlation with the mutagenicity assays
32   could be interpreted as rodent assays predominantly identifying non-genotoxic liver carcinogens,
33   this conclusion could be questioned because it is solely dependent on Salmonella mutagenicity
34   and additional genotoxic endpoints could conceivably shift the association between liver cancer
35   and genotoxicity towards a more positive correlation. As to questions of the usefulness of the
36   mouse bioassay, the two mutagenicity assays did not correlated with rat results either and an
37   important indicator for carcinogenicity would be lost.
38
39   Examination of tumor phenotype from TCE, DCA and TCA exposures in mice shows a
40   large heterogeneity which is also consistent with the heterogeneity observed in human HCC (see
41   Section 3.1.8., below). The heterogeneity of tumor phenotype has been correlated with survival
42   outcome and tumor aggressiveness in humans and in transgenic mouse models that share some of
43   the same perturbations in gene pathway expression (see Sections 3.1.8. and 3.2.1, below). An

1   examination of common pathway disturbances that may be common to all cancers and those of
2   liver tumors shows that there are pathways in common but that there is greater heterogeneity in
3   disturbance of hepatic pathways in cancer that may make is useful as a marker of disturbance of
4   cancer pathway that may lead to different targets of carcinogenicity depending on the cellular
5   context and target. Thus, although primate and human liver may not be as susceptible to HCC as
6   the rodent liver, the pathways leading to HCC in rodents and humans appear to be similar and
7   heterogeneous, with some indicative of other susceptible cellular targets for neoplasia in a
8   differing context.
9

3.1. State of Science for Cancer and Specifically Human Liver Cancer

3.1.1. Epigenetics and Disease States (Transgenerational Effects, Effects of Aging and Background changes)

15   Recently, Wood et al. (2007) published their work on "genomic landscapes" of human
16   breast and colorectal cancers that significantly forwards the understanding of "key events"
17   involved with induction of cancer. They state that there are ~ 80 DNA mutations that alter
18   amino acid in a typical cancer but that examination of the overall distribution these mutations in
19   different cancers of the same type leads to a new view of cancer genome landscapes: they are
20   composed of a handful of commonly mutated genes "mountains" but are dominated by a much
21   larger number of infrequently mutated gene "hills." "Statistical analyses suggested that most of
22   the ~ 80 mutation in an individual tumor were harmless and that <15 were likely to be
23   responsible for driving the initiation, progression, or maintenance of the tumor." "Historically
24   the focus of cancer research has been on the gene mountains, in part because they were the only
25   alterations that could be identified with available technologies. However, our data show that vast
26   majority of mutations in cancers do not occur in such mountains. This new view of cancer is
27   consistent with the idea that a large number of mutations, each associated with a small fitness
28   advantage, drive tumor progression. It is the "hills" and not the "mountains" that dominate the
29   cancer genomic landscape." The large number of "hills" actually reflects alterations in a much
30   smaller number of cell signaling pathways. Indeed, pathways rather than individual genes
31   appear to govern the course of tumorigenesis." "It is becoming increasingly clear that pathways
32   rather than individual genes govern the course of tumorigenesis. Mutations in any of several
33   genes of a single pathway can thereby cause equivalent increases in net cell proliferation." "This
34   new view of cancer is consistent with the idea that a large number of mutations, each associated
35   with a small fitness advantage, drive tumor progression." Thus, when pathways are altered the
36   same phenotype can arise from alterations in any of several genes.

37   Consistent with the arguments put forth by Wood et al. (2007) for mutations in cancer is
38   the additional insight into pathway alterations by epigenomic mechanisms which can act
39   similarly as mutation. Weidman et al. (2007) report that "cell phenotype is not only dependent
40   on its genotype but also on its unique epigenotype, which is shaped by developmental history
41   and environmental exposures. The human and mouse genome projects identified approximately
42   15,500 and 29,000 CpG islands, respectively. Hypermethylation of CpG-rich regions of gene
43   promoters inhibit expression by blocking the initiation of transcription. DNA methylation is also

involved in the allelic inactivation of imprinted genes, the silencing of genes on the inactive X chromosome, and the reduction of expression of transposable elements. Because epigenomic modifications are copied after DNA synthesis by DNMT1, they are inherited during somatic cell replication." "Inherited and spontaneous or environmentally induced epigenetic alterations are increasingly being recognized as early molecular events in cancer formation. Furthermore, such epigenetic alterations are potentially more adverse than nucleotide mutations because their effects on regional chromatin structure can spread, thereby affecting multiple genetic loci. Although tumor suppressor gene silencing by DNA methylation occurs frequently in cancer, genome-wide hypomethylation is one of the earliest events to occur in the genesis of cancer. Demethylation of the genome can lead to the reactivation of transposable elements, thereby altering the transcription of adjacent genes, the activation of oncogenes such as H-Ras, and biallelic expression of imprinted loci (e.g., loss of IGF2 imprinting)." Thus, epigenetic modification may be worse than mutation in terms of cancer induction.

Dolinoy et al. (2007) report on the role of environmental exposures on the epigenome, especially during critical periods of development and their role in adult disease susceptibility. They report that "aberrant epigenetic gene regulation has been proposed as a mechanism of action for non-genotoxic carcinogenesis, imprinting disorders, and complex disorders including Alzheimer's disease, schizophrenia, asthma, and autism. Epigenetic modifications are inherited not only during mitosis but also can be transmitted transgenerationally (Rakyan et al., 2002; Rakyan et al., 2003; Anway et al., 2005). The influence on environmental factors on epigenetic gene regulation may also persist transgenerationally despite lack of continued exposure in second, third, and fourth generations (Anway et al., 2005). Therefore if the genome is compared to the hardware in a computer, the epigenome is the software that directs the computer's operation." "The epigenome is particularly susceptible to deregulation during gestation, neonatal development, puberty and old age. Nevertheless, it is most vulnerable to environmental factors during embryogenesis because DNA synthetic rate is high, and the elaborate DNA methylation pattern and chromatin structure required for normal tissue development is established during early development." "83 imprinted genes have been identified in mice and humans with 29 or about one third being imprinted in both species. Since imprinted genes are functionally haploid, they are denied the protection from recessive mutations that diploidy would normally afford. Imprinted genes that have been linked to carcinogenesis include IGF2 (Bladder, lung, ovarian and others), IGF2R (breast, colon, lung, and others), and Neuronatin (pediatric leukemia)."

Bjornsson et al. (2008) recently reported that not only were time-dependent changes in global DNA methylation within the same individuals in 2 separate populations in widely separated geographic locations, these changes showed familial clustering in both increased and decreased methylation. These results were not only suggested to support the relationship of age-related loss of normal epigenetic patterns as a mechanism for late onset of common human diseases but also that losses and gains of DNA methylation observed over time in different individuals could contribute to disease with the example provided of cancer which is associated with both hypomethylation and hypermethylation through activation of oncogenes and silencing of tumor suppressor genes. The study also showed considerable interindividual age variation,

1   with differences accruing over time within individuals that would be missed by studies that
2   employed group averaging.
3
4   The review by Reamone-Buettner and Borlak (2007) provide insight into the role of non-
5   coding RNAs in diseases such as cancer. They report that "a large number of non-coding RNAs
6   (ncRNAs) play important role in regulating gene expressions, and advances in the identification
7   and function of eukaryotic ncRNAs, e.g., microRNAs and their function in chromatin
8   organization, gene expression, disease etiology have been recently reviewed. The regulatory
9   pathways mediated by small RNAs are usually collectively referred to as RNA interference
10 (RNAi) or RNA-mediated silencing. RNAi can be triggered by small double-stranded RNA
11 (dsRNA) either introduced exogenously into cells as small interfering siRNAs or that have been
12 produced endogenously from small non-coding RNAs known as microRNAs (miRNAs). The
13 dsRNAs are characteristically cleaved by the ribonucleas III-enzyme Dicer into 21- to 23 nt
14 duplexes and the resulting fragments base-pair with complementary mRNA to target cleavage or
15 to repress translation." "Two mechanisms exist of miRNA-mediated gene regulation,
16 degradation of the target mRNA, and translational repression. Whether one or the other of these
17 mechanisms is used depends on the degree of the complementary between the miRNA and target
18 mRNA. For a near perfect match, the Argonaute protein in the RNA-induced silencing complex
19 (RISC) cleaves the mRNA target, which is destined for subsequent degradation by ribonucleases.
20 In the situation of a less degree of complimentarity, commonly occurring in humans, the
21 translational repression mechanism is used to control gene expression. However, the exact
22 mechanism for translational inhibition is unclear." The varying degrees in complimentarity
23 would help explain the large number of genes that could be affected by miRNA and pleiotropic
24 response.
25
26   The review by Feinberg et al (2006) specifically addresses the epigenetic progenitor
27 origin of human cancer. They conclude that epigenetic alterations are ubiquitous and serve as
28 surrogate alterations for genetic change (oncogene activation, tumour-suppressor-gene
29 silencing), by mimicking the effect of genetic change. They report that:
30
31   Advances in characterizing epigenetic alterations in cancer include global alterations,
32   such as hypomethylation of DNA and hypoacetylation of chromatin, as well as gene-
33   specific hypomethylation and hypermethylation. Global DNA hypomethylation leads to
34   chromosomal instability and increased tumour frequency, which has been shown in vitro
35   and in vivo in mouse models, as well as gene-specific oncogene activation, such as R-ras
36   in gastric cancer, and cyclin D2 and maspin in pancreatic cancer. In addition, the
37   silencing of tumour-suppressor genes is associated with promoter DNA hypermethylation
38   and chromatin hypoacetylation, which affect divergent genes such as retinoblastoma 1
39   (RB1), p16 (also known as cyclin-dependent kinase inhibitor 2A (CDKN2A), von
40   Hippel-Lindau tumor suppressor (VHL), and MutL protein homologue (MLH1).
41
42   Genetic mechanisms are not the only path to gene disruption in cancer. Pathological
43   epigenetic changes - non-sequence-based alteration that are inherited through cell

division - are increasingly being considered as alternatives to mutations and chromosomal alterations in disrupting gene function. These include global DNA hypomethylation, hypermethylation and hypomethylation of specific genes, chromatin alterations and loss of imprinting. All of these can lead to aberrant activation of growth-promoting genes and aberrant silencing of tumour-suppressor genes.

Most CG dinucleotides are methylated on cytosine residues in vertebrate genomes. CG methylation is heritable, because after DNA replication the DNA methyltransferase 1, DNMT1, methylates unmethylated CG on the base-paired strand. CG dinucleotides within promoters within promoters tend to be protected from methylation. Although individual genes vary in hypomethylation, all tumours have shown global reduction of DNA methylation. This is a striking feature of neoplasia.

In addition to global hypomethylation, promoters of individual genes show increased DNA methylation levels. Hypermethylation of tumour-suppressor genes can be tumour-type specific. An increasing number of genes are found to be normally methylated at promoters but hypomethylated and activated in the corresponding tumours. These include R-RAs in gastric cancer, melanoma antigen family A, 1(MAGE1) in melanoma, maspin in gastric cancer, S100A4 in colon cancer, and various genes in pancreatic cancer.

Our genetic material is complexed with proteins in the form of histones in a one-to-one weight ratio. Core histones H2A, H2B, H3 and H4 form nucleosome particles that package 147 bp of DNA, and the linker histone H1 packages more DNA between core particles, forming chromatin. It is chromatin and not just DNA, that is the substrate for all processes that affect genes and chromosomes. In recent years, it has become increasingly evident that chromatin, like DNA methylation, can impart memory to genetic activity. There are dozens of post-translational histone modifications. Studies in many model systems have shown that particular histone modifications are enriched at sites of active chromatin (histone H3 and H4 hyperacetylation, lysing at 4 and H3 (H3-K4) dimethylation and trimethylation, and H3-K79 methylation) and others are enriched at sites of silent chromatin (H3-K9 and H3-K27 methylation). These and other histone modifications survive mitosis and have been implicated in chromatin memory.

Overproduction of key histone methyltransferases that catalyze the methylation of either H3-K4 or H3-K27 residues are frequent events in neoplasia. Global reductions in monoacetylated H4-K16 and trimethylated H4-K20 are general features of cancer cells.

Genomic imprinting is parent-of –origin-specific gene silencing. It results from a germ-line mark that causes reduced or absent expression of a specific allele of a gene in somatic cells of the offspring. Imprinting is a feature of all mammals affecting genes that regulate cell growth, behaviour, signaling, cell cycle and transport; moreover, imprinting is necessary for normal development.   Imprinting is important in neoplasia because both gynogenotes (embryos derived only from the maternal genetic complement) and

1   androgenotes (embryos derived only from the paternal genetic complement) form
2   tumours – ovarian teratomas, and hydtidiform moles/ choriocarcinomas, respectively.
3   Loss of imprinting (LOI) refers to activation of the normally silenced allele, or silencing
4   of the normally active allele, of an imprinted gene. LOI of the insulin-like growth factor
5   2 gene (IGF2) accounts for half of Wilms tumours in children. LOI of IGF2 is also a
6   common epigenetic variant in adults and is associated with a fivefold increased frequency
7   of colorectal neoplasia. LOI of IGF2 might cause cancer by increasing the progenitor cell
8   population in the kidney in Wilm's tumor and in the gastrointestinal tract in colorectal
9   cancer.
10
11   Feinberg et al. (2006) propose that epigenetic changes can provide mechanistic unity to
12   understanding cancer, they can occur earlier and set the stage for genetic alterations, and have
13   been linked to the pluripotent precursor cells form which cancers arise. "To integrate the idea of
14   these early epigenetic events, we propose that cancer arises in three steps; an epigenetic
15   disruption of progenitor cells, an initiating mutation and genetic and epigenetic pasticity."
16
17   The first step involves an epigenetic disruption of progenitor cells in a given organ or
18   system, which leads to a polyclonal precursor population of neoplasia-ready cells. These
19   cells represent a main target of environmental, genetic and age-dependent exposure that
20   largely accounts for the long latency period of cancer. Epigenetic disruption might
21   perturb the normal balance between undifferentiated progenitor cells and differentiated
22   committed cells within a given anatomical compartment, either in number or in their
23   capacity for aberrant differentiation, which provides a common mechanism of neoplasia.
24
25   All tumours show global changes in DNA methylation, and DNA methylation is clonally
26   inherited through cell division. Because the conventional genetic changes in cancer are
27   also clonal, global hypomethylation would have to occur universally, at the same moment
28   as the mutational changes, which seems unlikely. This suggests that global DNA
29   hypomethylation (and global reductions of specific histone modifications) precedes
30   genetic change in cancer. Similarly, hypermethylation of tumour-suppressor genes has
31   been observed in the normal tissue of patients in which the same gene is hypermethylated
32   in the tumour tissue. Recent data demonstrate LOI of IGF2 throughout the normal
33   colonic epithelium of patients who have LOI-associated colorectal cancer. LOI is
34   associated with increased risk of intestinal cancers in both humans and mice. A specific
35   change in the epithelium is seen in mice that are engineered to have biallelic expression
36   of IGF2 – a shift in the proportion of progenitor to differentiated cells throughout the
37   epithelium; a similar abnormality was observed in humans with LOI of IGF2.
38
39   The proposed existence of the epigenetically disrupted progenitors of cancer implies that
40   the earliest stages in neoplastic progression occur even before what a pathologist would
41   recognize as a benign pre-neoplastic lesion. Such alterations are inherently polyclonal.
42   This is in contrast with the widely accepted model of cancer as a monoclonal disorder
43   that arises from an initiating mutation- a model that was proposed and accepted when

1   little was known about epigenetic phenomena in cancer.

2

3   Thus, Feinberg et al. (2006) provide a hypothesis for the latency period of cancer and
4 suggest that epigenetic changes predate mutational ones in cancer. Tissues which look
5 phenotypically "normal" may harbor epigenetic changes and predispositions toward neoplasia.
6 In regard to what cells may be targets or epigenetic changes that can be "progenitor cells" in the
7 case of cancer, Feinberg et al. (2006) define such cell having "capacity for self-renewal and
8 pluripotency – over their tendency toward limited replicative potential and differentiation."
9 Within the liver, there are multiple cell types that would fit such a definition including those who
10 are considered "mature" (see Section 3.1.4, below). Feinberg et al. (2006) also note that
11 epigenetic states can be continuously modified to become heterogeneous at all states of the
12 neoplastic process.

13

14   Telomere erosion results in chromosome shortening and uncapped ends that begin to fuse
15   and the resulting dicentric chromosomes break at anaphase. DNA palindromes have
16   recently been found to form at high levels in cancer cells. Like telomere erosion, DNA
17   palindrome formation can lead to genetic instability by initiating bridge-breakage-fusion
18   cycles. However, it is not known how or exactly when palindromes form, although they
19   appear early in cancer progression. Epigenetic instability can also promote cancer
20   through pleiotropic alterations in the expression of genes that modify chromatin.

21

22   Epigenetic changes are reversible but the changes can initiate irreversible genetic
23   changes. Permanent epigenetic changes can have an epigenetic basis. On a background of
24   cancer-associated epigenetic instability, the effects of mutations in oncogenes and tumour
25   –suppressor genes might be exacerbated. Therefore the risk of developing malignancy
26   would be much higher for a given mutations event if it occurred on the background of
27   epigenetic disruption.

28

29   The environmental dependence of cancer fits an epigenetic model generally for human
30   disease – the environment might influence disease onset not simply through mutational
31   mechanisms but in epigenetically modifying genes that are targets for either germline or
32   acquired mutation; that is, by allowing genetic variates to be expressed. Little is known
33   about epigenetic predispositions to cancer, but a recent twin study indicates that, similar
34   to cancer risk, global epigenetic changes show striking increase with age.

35

36   Environmental insults might affect the expression of tumour-progenitor genes, leading to
37   both genetic and epigenetic alterations. Liver regeneration after tissue injury leads to
38   widespread hypomethylation and hypermethylation of individual genes; both of these
39   epigenetic changes occur in cancer.

40

41   In regard to the implications of epigenomic changes and human susceptibility to toxic
42 insult, the review by Szyf (2007) provides additional insights.

43

1   The basic supposition in the field has been that the interindividual variations in response
2   to xenobiotic are defined by genetic differences and that the main hazard anticipated at
3   the genomic level from xenobiotic is mutagenesis or physical damage to DNA. In
4   accordance with this basic hypothesis, the main focus of attention in pharmacogenetics
5   has been on identifying polymorphisms in genes encoding drug metabolizing enzymes
6   and receptors. New xenobiotics were traditionally tested for their genotoxic effects.
7   However, it is becoming clear that epigenetic programming plays an equally important
8   role in generating interindividual phenotypic differences, which could affect drug
9   response. Moreover, the emerging notion of the dynamic nature of the epigenome and its
10 responsibility to multiple cellular signaling pathways suggest that it is potentially
11 vulnerable to the effects of xenobiotics not only during critical period in development but
12 also later in life as well. Thus, non-genotoxic agents might affect gene function through
13 epigenetic mechanisms in a stable and long-term fashion with consequences, which might
14 be indistinguishable form the effects of physical damage to the DNA. Epigenetic
15 programming has the potential to persist and even being transgenerationally transmitted
16 (Anway et al., 2005) and this possibility creates a special challenge for toxicological
17 assessment of safety of xenobiotics. Any analysis of interindividual phenotype diversity
18 should therefore take into account epigenetic variations in addition to genetic sequence
19 polymorphisms. Whereas, a germ-line polymorphism is a static property of an individual
20 and might be mapped in any tissue at any point in life, epigenetic differences must be
21 examined at different time points and at diverse cell types.
22
23   Karpinets and Foy (2005) propose that epigenetic alterations preceed mutations and that
24 succeeding mutations are not random but in response to specific types of epigenetic changes the
25 environment has encouraged. This mechanism was also suggested as to both explain the delayed
26 effects of toxicant exposure and the bystander effect of radiation on tumor development, which
27 are inconsistent with the accepted mechanism of direct DNA damage. "In a study of ionizing
28 radiation, non-irradiated cells acquired mutagenesis through direct contact with tclls whose
29 nuclei had previously been irradiated with alpha-particles (Zhou et al., 2003). Molecular
30 mechansms underlying these experimental findings are not known but it is believed that it may
31 be a consequence of bystander interactions involving intercellular signaling and production of
32 cytokines (Lorimore et al., 2003)."
33
34   Caldwell and Keshava (2006) report that "aberrant DNA methylation has emerged in
35 recent years as a common hallmark of all types of cancers with hypermethylation of the promoter
36 region of specific tumor suppressor genes and DNA repair genes leading to their silencing (an
37 effect similar to their mutation), and genomic hypomethylation (Ballestar and Esteller, 2002;
38 Berger and Daxenbichler, 2002; Herman et al.,1998; Pereira et al. 2004; Rhee et al., 2002).
39 Whether DNA methylation is a consequence or cause of cancer is a long-standing issue
40 (Ballestar and Esteller, 2002). Fraga et al. (2004, 2005) report global loss of monoacetylation
41 and trimethylation of histone H4 as common a hallmark of human tumor cells but suggest
42 genomone-wide loss of 5-methylcytosine (associated with the acquisition of a transformed
43 phenotype) does not exist as a static predefined value throughout the process of carcinogenesis

1 but as a dynamic parameter (i.e., decreases are seen early and become more marked in later
2 stages)."
3
3.1.2. Emerging Technologies, DNA and siRNA, miRNA Microarrays– promise and limitations for MOA
6
7 Currently new approaches are emerging for the study of changes in gene expression and
8 protein production induced by chemical exposure that could be related to their toxicity and serve
9 as an anchor for determining similar patterns between rodent models and human diseases or risks
10 of chemically-induced health impacts. Such approaches have the promise to extend the
11 definitions of "genotoxic" and "non-genotoxic" effects which with the advent of epigenomic
12 study have become obsolete as they assume only alteration of the DNA sequence is important in
13 cancer induction and progression. However, not only is phenotypic anchoring an issue in
14 regard to the differing cell types, regions, and lobes of the liver (see Section 1.2, above), it is also
15 an issue for overall variability of response between animals and is critical for interpretation of
16 microarray and other genomic database approaches. As shown in the discussions of TCE effects
17 in animal models, TCE treatment resulted in a large variability in response between what are
18 supposed to be relatively homogeneous genetically similar animals and there was an apparent
19 difference in response between studies using the same paradigm. It is important that as varying
20 microarray approaches and analyses of TCE toxicity or of potential MOAs are published, the
21 issue of phenotypic anchoring at the cellular to animal level is addressed. Several studies of
22 TCE microarray results and those of PPARα agonists have been reported in the literature in an
23 attempt to discern MOAs. Issues related to conduct of these experiments and interpretation of
24 their results are listed below.
25
26 Perhaps one of the most important studies of this issue has been reported by Baker et al.
27 (2004). The ILSI HESI formed a hepatotoxicity working group to evaluate and compare
28 biological and gene expression responses in rats exposed to well-studied hepatotoxins (Clofibrate
29 and methapyrilene), using standard experimental protocol and to address the following issues: a)
30 how comparable are the biological and gene expression data from different laboratories running
31 identical in vivo studies b) how reproducible are the data generated across laboratories using the
32 same microarray platform c) how do data compare using different microarray platforms; d) how
33 do data compare using RNA from pooled and individual animals; e) do the gene expression
34 changes demonstrate time- and dose-dependent responses that correlate with known biological
35 markers of toxicity? (Baker et al., 2004). The rat model studied was the male Sprague-Dawley
36 rat (57 or 60-66 days of age) exposed to 250 or 25 mg/kg/day Clofibrate for 1, 3 or 7 days. Two
37 separate *in vivo* studies were conducted: one at Abbott Laboratories and on at GSK (UK). There
38 was a difference in biological response between the two laboratories.   The high dose (250
39 mg/kg/day) group at day 3 had a 15% increase in liver weight relative to body weight in the GSK
40 study, compared with a 3% liver weight increase in the Abbott study. At 7-days, there was a
41 31% liver weight increase in the GSK study and 15% in the Abbott study. Observed changes in
42 clinical chemistry parameters also indicated difference in the biological response of the *in vivo*
43 study concordant with difference in liver weight. A significant reduction in total cholesterol

1   levels was seen in the GSK study at the high dose for all time points. However, the Abbott study
2   demonstrated a significant reduction only at one dose and time point. The incidence of mitotic
3   figures also differed between the labs. In both studies there was a 2-3 times greater Acyl-CoA
4   enzyme (ACOX) activity at the high dose but no difference from control in the low dose. Again
5   the GSK lab gave greater response. For microarrays, GSK and ULR pooled samples from each
6   treatment group of 4 animals. U.S. EPA did some of the microarray analyses as well as GSK
7   and ULR (GSK in UK). It is apparent that although the changes in genes were demonstrated by
8   both laboratories, there were quantitative differences in the fold change values observed between
9   the two sites.
10
11   The U.S. EPA analyzed gene expression in individual RNA samples obtained from day 7
12 high and low-dose animals that had been treated at Abbot. GSK (US) and ULR analyzed gene
13 expression in pooled RNA from day 7 high and low dose animals treated at GSK (UK). Gene
14 expression data from individual animal samples indicated that 7 genes were significantly
15 upregulated (maximum of 7.2-fold) and 12 were down regulated (maximum of 4.3-fold decrease)
16 in the high dose group. The low dose group generated only one statistically significant gene
17 expression change, namely heat shock protein 70 (HSP70), In comparison, expression changes
18 in the 7-day pooled high-dose samples analyzed by GSK (U.S.) ranged from 43.3-fold to a 3.5-
19 fold decrease. Changes in these same samples analyzed by ULR ranged from a 4.9 fold increase
20 to a 4.3-fold decrease. As an example, the microarray fold change at 7-day 250 mg/kg/day
21 Clofibrate showed a 3.8-fold increase for US EPA individual animals sampled, and 2.2-fold
22 increase for pooled samples by ULR, and a 20.3-fold increase in pooled samples by GSK (US)
23 for CYP4A1 (Baker et al., 2004). Thus, these results show a very large difference not only
24 between treatment groups but between pooled an non-pooled data and between labs analyzing
25 the same RNA.

26   Not only was there a difference in DNA microarray results but a comparison of gene
27 expression data from day 7 high-dose samples obtained using quantitative realtime PCR versus
28 data generated using cDNA microarrays is shown a quantitative difference but qualitative similar
29 patterns. Although both methods of quantitative real time PCR on the pooled sample showed the
30 PPARα gene to be down regulated, the GSK (IS) pooled sample microarray analysis indicated
31 upregulation; the URL pooled and U.S. EPA individual microarray analyses showed no change.
32 The microarray for PPARα at 7-day 250 mg/kg/day Clofibrate showed no change for individual
33 animals (U.S EPA), no change for pooled samples (ULR) and upregulation of 1.8-fold value for
34 pooled samples for GSK(US).   The quantitative real time PCR on the pooled sample using
35 Taqman gave a 4.5-fold down regulation and using SYBR Green gave a 1.2-fold down
36 regulation of PPARα.

37   Baker et al. (2004) reported that the pooling of samples for microarray analysis has been
38 used in the past to defray the cost of microarray experiments, reduce the effect of biological
39 variation, and in some cases overcome availability of limiting amounts of tissues. Unfortunately
40 this approach essentially produced a sample size (n) of one animal. Repeated microarray
41 experiments with such pooled RNA produces technical replicates as opposed to true biological
42 replicates and thus does not allow calculation of biologically significant changes in gene

1   expression between different dose groups or time points. Another possible consequence of
2   pooling is to mask individual gene changes and leave open the possibility of introducing error
3   due to individual outlier responses.

4   Woods et al. (2007a) note that "because toxicogenomics is a relatively novel technology,
5   there are a number of limitations that must be resolved before array data is widely accepted.
6   Microarray studies have been touted as being highly sensitive for detecting toxic responses at
7   much earlier time points and/or lower doses than histopathology, clinical chemistry or other
8   traditional toxicological assays can detect. However, based on the nature of the assay,
9   measurements of extreme levels of gene expression – low or high –are thought to be unreliable.
10 Also the reproducibility of microarray experiments has raised concerns. "Batch effects" based
11 on the day, user, and laboratory environment have been observed in array datasets. To address
12 these concerns, confirmation of microarray-derived gene expression profiles is typically
13 performed using quantitative real time polymerase chain reaction (RT-PCR) or Northern blot
14 analysis."

15

16   In addition to the issues raised above, Waxman et al. (2007) raise issues regarding how
17 quantitative realtime PCR experiments are conducted. They state that cancer development
18 affects almost all pathways and genes including the "housekeeping" genes, which are involved in
19 the cell's common basic functions [e.g., glyceraldehyde-3-phosphate dehydrogenase (GADPH),
20 beta actin (ACTB), TATA-binding protein (TBP), ribosomal proteins (RP), and many more].
21 However, "many of these genes are often used to normalize quantitative real-time RT-
22 PCR(qPCR) data to account for experimental differences, such as differences in RNA quantity
23 and quality, the overall transcriptional activity and differences in cDNA synthesis. GADPH and
24 ACTB are most commonly used for normalization, including studies of cancer. Waxman et al
25 (2007) suggest that despite the fact that it has been shown that these genes are differentially
26 expressed in cancers, including colorectal-, prostate-, and bladder-cancer, some qPCR studies on
27 hepatocellular carcinoma used GAPDH or ACTB for normalization. Since many investigations
28 on cancer include multiple comparisons, and analyze different stages of the disease, such as
29 normal tissue, pre-neoplasm, and consecutive stages of cancer, "it crucial to find an appropriate
30 gene for normalization" whose expression is constant throughout all disease stage and not
31 response to treatment. For liver cancers associated with exposure to hepatitis C virus (HCV),
32 Waxman et al (2007) reported that differing states, including preneoplastic lesions (cirrhosis and
33 dysplasia) and consecutive stages of hepatocellular carcinoma, had differential expression of
34 "housekeeping" genes and that using them for normalization had an effect on the fold change of
35 qPCR data and on the general direction (up or down) of differentially expressed genes. For
36 example, GAPDH was strongly upregulated in advanced and very advanced stages of
37 hepatocellular carcinoma (in some samples up to 7-fold) and ACTB was up-regulated 2- to 3-
38 fold in many advanced and very advanced tumor samples. Waxman et al. (2007) conclude that
39 "microarray data are known to be highly variable. Due to its higher dynamic range qPCR is
40 thought to be more accurate and therefore is often used to corroborate microarray results.
41 Mostly, general direction (up and down-regulation) and rank order of the fold-changes are
42 similar, but the levels of the fold changes of microarray experiments differ compared to qPCR

1   data and show a marked tendency of being smaller. This effect is more pronounced as the fold
2   change is very high."
3
4   In relation to use of gene expression and indicators of cancer causation, Volgelstein and
5   Kinzler (2004) make important points regarding their use:
6
7   Levels of gene expression are unreliable indicators of causation because disturbance of
8   any network invariably leads to a multitude of such changes only peripherally related to
9   the phenotype. Without better ways to determine whether an unmutated but interesting
10   candidate gene has a causal role in neoplasia, cancer researchers will likely be spending
11   precious time working on genes only peripherally related to the disease they wish to
12   study.
13
14   This is important caveat for gene expression studies for MOA that are "snapshots in time"
15   without phenotypic anchoring and even more applicable to experimental paradigms where there
16   is ongoing necrosis or toxicity in addition to gene changes that may or may not be associated
17   with neoplasia.
18
19   For an endpoint that is not as complex as neoplasia, there are issues regarding uses of
20   microarray data. In regard to the determination of acute liver toxicity caused by one of the most
21   studied hepatotoxins, acetaminophen, and its correlation with microarray data, Beyer et al.
22   (2007) also have reported the results of landmark study examining issues regarding use of this
23   approach. "The biology of liver and other tissues in normal and disease states increasingly is
24   being probed using global approaches such as microarray transcriptional profiling. Acceptance
25   of this technology is based principally on a satisfactory level of reproducibility of data among
26   laboratories and across platforms. The issue of reproducibility and reliability of genomics data
27   obtained from similar (standardized) biological experiments performed in different laboratories
28   is crucial to the generation and utility of large databases of microarray results. While several
29   recent studies uncovered important limitation of expression profiling of chemical injury to cells
30   and tissues (Baker et al 2004; Beekman et al 2006; Ulrich et al 2004), determining the effects of
31   intralaboratory variables on the reproducibility, validity, and general applicability of the results
32   that are generated by different laboratories and deposited into publicly available databases
33   remains a gap." "The National Institutes of Environmental Health Sciences (NIEHS) established
34   the Toxicogenomics Research Consortium to apply the collective and specialized expertise from
35   academic institutions to address issues in integrating gene expression profiling, bioinformatics,
36   and general toxicology. Key elements include developing standardized practices for gene
37   expression studies and conducting systematic assessments of the reproducibility of traditional
38   toxicity endpoints and microarray data within and among laboratories. To this end the
39   consortium selected the classical hepatotoxicant acetaminophen (APAP) for its proof of concept
40   experiments. Despite more than 30 years of research on APAP, we are far from a complete
41   understanding of the mechanisms of liver injury, risk factors, and molecular markers that predict
42   clinical outcome after poisoning. APAP–induced hepatotoxicity was performed at seven
43   geographically dispersed Centers. Parallel studies with N-acetyl-m-aminophenol (AMAP), the

1   non-hepatotoxic isomer of APAP, provided a method to isolate transcripts associated with
2   hepatotoxicity" (Beyer et al., 2007).
3
4   Beyer et al identified potential sources of interlaboratory variability when microarray
5   analyses were conducted by one laboratory on RNA samples generated in different laboratories
6   but using the same experimental paradigm and source of animals. Toxic injury by APAP
7   showed variability across Centers and between animals [e.g., % liver affected by necrosis (<20%
8   to 80% at one time period and 0% to 60% at another), control animal serum ALT (3-fold
9   difference), and in glutathione depletion (<5% to > 60%) between centers]. There was
10 concordance between APAP toxicity as measured in individual animals (rather than expressed as
11 just a mean with SE) and transcriptional response. Of course the variability between gene
12 platforms and processing of the microarrays had been reduced by using the same facility to do all
13 of the microarray analyses. However, the results show that phenotypic anchoring of gene
14 expression data is required for biologically meaningful meta-analysis of genomic experiments.
15
16   Woods et al. (2007a) note that "improvements should continue to be made on statistical
17 analysis and presentation of microarray data such that it is easy to interpret. Prior to the current
18 advances in bioinformatics, the most common way of reporting results of microarray studies
19 involved listing differentially expressed genes, with little information about the statistical
20 significance or biological pathways with which the genes are associated." However, there are
21 issues with the use of "Classifiers" or predictive genomic computer programs based on genes
22 showing altered expression in association with the observed toxicities. "Although these metrics
23 built on different machine learning algorithms could be useful in estimating the severity of
24 potential toxicities induced by compounds, the applications of these classifiers in understanding
25 the mechanisms of drug-induced toxicity are not straightforward. In particular this approach is
26 unlikely to distinguish the upstream causal genes from the downstream responsive genes among
27 all the genes associated with an induced toxicity. Without knowledge of the causal sufficiency
28 order, designing experiments to test predicted toxicity in animal models remains difficult" (Dai
29 et al., 2007). Ulrich (2003) states limitation of microarray analysis to study nuclear receptors
30 (e.g., PPARα). "Nuclear receptors comprise a large group of ligand-activated transcription
31 factors that control much of cellular metabolism. Toxicogenomics is the study of the structure
32 and output of the entire genome as it related and responds to adverse xenobiotic exposure.
33 Traditionally, the genes regulated by nuclear receptors in cells exposed to toxins have been
34 explored at the mRNA and protein levels using northern and western blotting techniques.
35 Though effective when studying the expression of individual genes, these approaches do not
36 enable the understanding of the myriad of genes regulated by individual receptors or of the
37 crosstalk between receptors." "Discovery of the multiple genes regulated by each receptor type
38 has thus been driven by technological advances in gene expressional analysis, most commonly
39 including differential display, RT-PCR and DNA microarrays., and in the development or
40 receptor transgenic and knockout animal models. There is much cross talk between receptors
41 and many agonists interact with multiple receptors. Off target effects cannot be predicted by
42 target specificity. Though RCR can affect transcription directly, much of its effects are exerted
43 through heterodimeric binging with other nuclear receptors (PXR, CAR, PPARα, PPARγ, FXR,

1 LXR, TR)" (Ulrich, 2003).
2
3   Another tool recent developed is gene silencing by introduction of siRNA. Dai et al.
4 (2007) note issues involved in the siRNA to change gene expression for exploration of MOA etc.
5 to include the potential of off-target effects, incomplete knockdown, and non-targeting of splice
6 variants by the selected siRNA sequence. Using knockdown of PPARα in mice, Dai et al. (2007)
7 report "PPARα knockdown was variable between mice ranging from ~ 80 % knockdown to little
8 or no knockdown and that differing siRNAs gave different patterns of gene expression with some
9 grouped with PPARα -/- null mice but others grouped with expression patterns of mice injected
10 with control siRNA or Ringers buffer alone and showing no PPARα knockdown. Dai et al
11 concluded that it is possible that it is the change in PPARα levels that is important for perturbing
12 expression of genes modulated by PPARα rather than the absolute levels of PPARα. Not only is
13 the finding of variability in knockdowns by siRNA technologies important but The finding that
14 level of PPAR is not necessarily correlated with function and that it could be the change and not
15 absolute level that matters in modulation in gene expression by PPARα is of importance as well.
16 How an animal responds to decreased PPARα function may also depend on its gender. Dai et al.
17 (2007) observed more dramatic phenotypes in female vs. male mice treated with siRNA and
18 noted that in aged PPARα -/- mice, Costet et al. (1998) have reported sexually dimorphic
19 phenotypes including obesity and increased serum triglyceride levels in females, and steatosis
20 and increased hepatic triglyceride levels in males.
21
22   In regard to the emerging science and preliminary reports of the effects of microRNA as
23 oncogenes and tumor suppressors and of possible importance to hypothesized MOAs for liver
24 cancer, the same caveats as described for DNA microarray analyses all apply along with
25 additional uncertainties. miRNAs repress their targeted mRNAs by complementary base pairing
26 and induction of the RNA interference pathway. Zhang et al. (2007) report Northern blot
27 detection of gene expression at the mRNA level and its correlation with miRNA expression in
28 cancer cells as well as realtime PCR. These PCR-based analyses quantify miRNA precursors
29 and not the active mature miRNAs. However, they report that the relationship between pri-
30 miRNA and mature miRNA expression has not been thoroughly addressed and is critical in order
31 to use real time PCR analysis to study the function of miRNAs in cancers. They go on to state
32 that "although Northern Blotting is a widely used method for miRNA analysis, it has some
33 limitations, such as unequal hybridization efficiency of individual probes and difficulty in
34 detecting multiple miRNAs simultaneously. For cancer studies, it is important to be able to
35 compare the expression pattern of all known miRNAs between cancer cells and normal cells.
36 Thus, it is better to have methods which detect all miRNA expression at a single time."
37 "Although Northern blot analysis, real-time PCR, and miRNA microarray can detect the
38 expression of certain miRNAs and determine which miRNAs may be associated with cancer
39 formation, it is difficult to determine whether or not miRNAs play a unique role in cancers. Also
40 these techniques cannot directly determine the correlation between mRNA expression levels and
41 whether the up-regulation or down-regulation of certain miRNAs is the cause of cancer or a
42 downstream effect of the disease." "Many miRNA genes have been found that are significantly
43 overexpressed in different cancers. All of them appear to function as oncogenes; however, only

1   a few of them have been well characterized." Zhang et al. (2007) suggest that bioinformatic
2   studies indicate that numerous genes are the targets of miR-17-92: more than 600 for miR-19a
3   and miR-20, two members of the miR-17-92 cluster." Cho et al. (2007) state that "though more
4   than 530 miRNAs have been identified in human, much remains to be understood about their
5   precise cellular function and role in the development of diseases." "Although each miRNA can
6   control hundreds of target genes, it remains a great challenge to identify the accurate miRNA
7   targets for cancer research." Thus, miRNAs have multiple target so like other transcription
8   factors may have pleotropic effects that are cell, timing, and context specificity.
9
10   Vogelstein and Kinzler (2004) go on to state "in the last decade many important gene
11   responsible for the genesis of various cancers have been discovered." Most importantly they and
12   other suggest that pathways rather than individual gene expression should be the focus of study.
13   As a specific example, Volgelstein and Kinzler note "another example of the reason for focusing
14   on pathways rather than individual genes has been provided by studies of TP53 tumor-suppressor
15   gene. The p53 protein is a transcription factor that normally inhibits cell growth and stimulates
16   cell death when induced by cellular stress. The most common way to disrupt the p53 pathway is
17   through a point mutation that inactivates its capacity to bind specifically to its cognate
18   recognition sequence. However, there are several other ways to achieve the same effects,
19   including amplification of the MDM2 gene and infection with DNA tumor viruses whose
20   products bind to p53 and functionally inactivate it."
21
22   In regard to cellular anchoring for gene expression or pathway alterations associated with
23   cancer and the importance of "context" of gene expression changes, Vogelstein and Kinzler
24   (2004) give several examples. "In solid tumors the important of the interactions between stroma
25   and epithelium is becoming increasingly recognized (e.g., the importance of the endothelial
26   cell)." "One might expect that a specific mutation of a widely expressed gene would have
27   identical or at least similar effects in different mammalian cell types. But this is not in general
28   what is observed. Different effects of the same mutation are not only found in distinct cell types;
29   difference can even be observed in the same cell types, depending on when the mutation
30   occurred during the tumorigenic process. The RAS gene mutations provide informative
31   examples of these complexities. *KRAS2 g*ene mutation in normal pancreatic duct cells seem to
32   initiate the neoplastic process, eventually leading to the development of pancreatic cancer. The
33   same mutations occurring in normal colonic or ovarian epithelial cells lead to self-limiting
34   hyperplastic or borderline lesions that do not progress to malignancy. In many human and
35   experimental cancers, *RAS* genes seem to function as oncogenes. But *RAS* genes can function as
36   suppressor genes under other circumstances, inhibiting tumorigenesis after administration of
37   carcinogens to mice. These and similar observation on other cancer genes are consistent with the
38   emerging notion that signaling molecules play multiple roles at multiple time, even in the same
39   cell type. However, the biochemical bases for such variations among cancer cells are almost
40   unknown."
41
42   In regard to the major pathways and mediators involved in cancer several investigators
43   have reported a coherent set that are involved in many types of cancers. Vogelstein and Kinzler

1　(2004) note that major pathways and mediators include p53, RB, WNT, E-cadherin, GL1, APC,
2　ERK, RAS:GTP, P13K,SMAD, RTK BAD, BAX, and H1F1. In regard to coherence and site
3　concordance between animal and human data, the disturbance of a pathway in one species may
4　result in the different expression of tumor pattern in another but both linked to a common
5　endpoint of cancer. Thus, pathways rather than a single mutation should be the focus of MOA
6　and cancer as several actions can be manifested by one pathway or change at one time that lead
7　to cancer.
8
9　　　Vogelstein and Kinzler (2004) also note that pathways that are common to "cancer" are
10　also operative in liver cancer where, as a heterogeneous disease, multiple pathways have been
11　implicated in differing manifestations of this disease. Thus, liver cancer may be an example its
12　multiple forms are analogous to differing sites being affected by common pathways that are
13　affected in "cancer." Pathway concordance may not always show up as site concordance as
14　expression of cancer between species. Liver cancer may be the example where many pathways
15　can lead a cancer that is characterized by its heterogeneity.

16　**3.1.3. Etiology, Incidence and Risk Factors for HCC**
17
18　　　The review article of Farazi and DePinho (2006) provides and excellent summary of the
19　current state of human liver cancer in terms of etiology and incidence. The 5-year survival rate
20　of individuals with liver cancer in the United States is only 8.9% despite aggressive conventional
21　therapy with lethality of liver cancer due in part from its resistance to existing anticancer agents,
22　a lack of biomarkers that can detect surgically respectable incipient disease, and underlying liver
23　disease that limits the use of chemotherapeutic drugs. Chen et al. (2002) report that surgical
24　resection is considered the only "curative treatment" but > 80 of patients have widespread
25　hepatocellular carcinoma (HCC) at the time of diagnosis and are not candidates for surgical
26　treatment. Among patients with localized HCC who undergo surgery, 50% suffer a recurrence.
27　Primary liver cancer is the fifth most common cancer worldwide and the third most common
28　cause of cancer mortality. Hepatocellular carcinoma (HCC) accounts for between 85% and 90%
29　of primary liver cancers (El-Serag and Rudolph, 2007). Seitz and Stickel (2006) report that
30　epidemiological data from the year 2000 indicate that more than 560,000 new cases of HCC
31　occurred worldwide, accounting for 5.6% of all human cancers and that HCC is the fifth most
32　common malignancy in men and the eighth in women. Overall, incidence rates of HCC are
33　higher in males compared to females. In almost all populations, males have higher liver cancer
34　rates than females, with male:female ratios usually averaging between 2:1 and 4:1 and the largest
35　discrepancies in rates (>4:1) found in medium-risk European populations (El-Serag and Rudolph,
36　2007). Experiments show a 2- to 8-fold of control HCC development in male mice as well
37　supporting the hypothesis that androgens influence HCC progression rather than sex-specific
38　exposure to risk factors (El-Serag and Rudolph, 2007). El-Serag and Rudolph (2007) also report
39　that "in almost all areas, female rates peak in the age group 5 years older than the peak age
40　group for males. In low risk population (e.g., U.S.) the highest age-specific rates occur among
41　persons aged 75 and older. A similar pattern is seen among most high-risk Asian populations.
42　In contrast male rats in high-risk African populations (e.g., Gambia) ten to peak between ages 60

1   and 65 before declining, whereas female rates peak between 65 and 70 before declining."

3   Age adjusted incidence rates for HCC are extremely high in East and Southeast Asia and
4   in Africa but in Europe, there is a gradually decreasing prevalence from South to North. HCC
5   incidence rates also vary greatly among different populations living in the same region and vary
6   by race (e.g., for all ages and sexes in the US, HCC rates are 2 times higher in Asian than in
7   African Americans, whose rates are 2 times higher than those in whites) ethnic variability likely
8   to include differences in the prevalence and acquisition time of major risk factors for liver
9   disease and HCC (El-Serag and Rudolph, 2007). Worldwide HCC incidence rate doubled during
10   the last two decades and younger age groups are increasingly affected (El-Serag, 2004). The
11   high prevalence of HCC in Asia and Africa may be associated with widespread infection with
12   hepatitis B (HBV) and hepatitis C (HCV) but other risk factors include chronic alcohol misuse,
13   non alcoholic fatty liver disease (NAFLD), tobacco, oral contraceptives, and food contamination
14   with aflatoxins (Seitz and Stickel, 2006). El-Serag and Rudolph (2007) report HCC to be the
15   fastest growing cause of cancer-related death in men in the United States with age-adjusted HCC
16   incidence rates increasing more than 2-fold between 1985 and 2002 and that, overall, 15%-50%
17   of HCC patients in the United States have not established risk factors.

19   Although liver cirrhosis is present in a large portion of patients with HCC, it is not always
20   present. Fattrovich et al (2004) report that "differences of geographic area, method of
21   recruitment of the HCC cases (medical or surgical) and the type of material studied (liver biopsy
22   specimens, autopsy, or partial hepatectomies) may account for the variable prevalence of HCC
23   without underlying cirrhosis (7% to 54%) quoted in a series of studies. Percutaneous liver
24   biopsy specimens are subject to sampling error. However, only a small proportion of patients
25   with HCC without cirrhosis have absolutely normal liver histology, the majority of them
26   showing a range of fibrosis intensity from no fibrosis are all to septal and bridging fibrosis,
27   necroinflammation, steatosis, and liver cell dysplasia."

29   Farazi and DePinho (2006) note that for diabetes, a higher indices of HCC has been
30   described in diabetic patients with no previous history of liver disease associated with other
31   factors. El-Serag and Rudolph (2007) report that in their study of VA patients (173,643 patients
32   with and 650,620 patients without diabetes), that HCC incidence doubled among patients with
33   diabetes and was higher among those with a longer follow-up of evaluation. "Although most
34   studies have been conducted in low HCC rate areas, diabetes also has been found to be a
35   significant risk factor in areas of high HCC incidence such as Japan. Taken together, available
36   data suggest that diabetes is a moderately strong risk factor for HCC."

38   Non-alcoholic fatty liver disorders (NAFLD) and non-alcoholic steatohepatitis contribute
39   to the development of fibrosis and cirrhosis and therefore might also contribute to HCC
40   development. The pathogenesis of NAFLD includes the accumulation of fat in the liver which
41   can lead to reactive oxygen species in the liver with necrosis factor α (TNFα) elevated in
42   NAFDL and alcoholic liver disease (Seitz and Stickel 2006). Abnormal liver enzymes not due to
43   alcohol, viral hepatitis, or iron overload are present in 2.8% to 5.5% of the US general population

1   and may be due to NAFLD in 66% to 90% of cases (Adams and Lindor, 2007). Primary NAFLD
2   occurs most commonly and is associated with insulin-resistant states, such as diabetes and
3   obesity with other conditions associated with insulin resistance, such as polycystic ovarian
4   syndrome and hypopituitarism also associated with NAFLD (Adams and Lindor, 2007). The
5   steatotic liver appears to be susceptible to further hepatotoxic insults, which may lead to
6   hepatocyte injury, inflammation, and fibrosis, but the mechanisms promoting progressive liver
7   injury are not well defined (Adams and Lindor, 2007). Substrates derived from adipose tissue
8   such as FFA, TNF-α, leptin, and adiponectin have been implicated with oxidative stress
9   appearing to be important leading to subsequent lipid peroxidation, cytokine induction, and
10 mitochondrial dysfunction.   Liver disease was the third leading cause of death among NAFLD
11 patients compared to the 13th leading cause among the general population, suggesting that liver-
12 related mortality is responsible for a proportion of increased mortality risk among NAFLD
13 patients (Adams and Lindor, 2007).
14
15   The relative risk for HCC in type 2 diabetics has been reported to be approximately 4 and
16 increases to almost 10 for consumption of more than 80 g of alcohol per day (Hassan et al.,
17 2002). El-Serag and Rudolph (2007) report that "it has been suggested that many cryptogenic
18 cirrhosis and HCC cases represent more severe forms of nonalcoholic fatty liver disease
19 (NAFLD), namely nonalcoholic steato hepatitis (NASH). Studies in the United States evaluating
20 risk factors for chronic liver disease or HCC have failed to identify HCV, HBV, or heavy alcohol
21 intake in a large proportion of patients (30-40%). Once cirrhosis and HCC are established, it is
22 difficult to identify pathologic features of NASH. Several clinic-based controlled studies have
23 indicated that HCC patients with cryptogenic cirrhosis tend to have clinical and demographic
24 features suggestive of NASH (predominance of women, diabetes, and obesity) as compared with
25 age- and sex-matched HCC patients of well defined vial or alcoholic etiology. The most
26 compelling evidence for an association between NASH and HCC is indirect and come from
27 studies examining HCC risk with 2 conditions strongly associated with NASH: obesity and
28 diabetes. In a large prospective cohort in the US, followed up for 16 years, liver cancer mortality
29 rates were 5 times greater among men with the greatest baseline body mass index (range 35-40)
30 compared with those with a normal body mass index. In the same study, the risk of liver cancer
31 was not as increase in women, with a relative risk of 1.68. Two other population-based cohort
32 studies from Sweden and Denmark found excess HCC risk (increased 2- to 3-fold) in obese men
33 and women compared with those with a normal body mass index." "Finally, liver disease occurs
34 more frequently in those with more severe metabolic disturbances, with insulin resistance itself
35 shown to increase as the disease progresses. Several developed countries most notably the
36 United States, are in the midst of a burgeoning obesity epidemic. Although the evidence linking
37 obesity to HCC is relatively scant, even small increase in risk related to obesity could translate
38 into a large number of HCC cases." Thus, even a small increase in risk related to obesity could
39 result in a large number of HCC cases and the latency of HCC may make detection of increased
40 HCC risk not detectable for several years.
41
42   Other factors are involved as not every cirrhotic liver progresses to HCC. Seitz and
43 Stickel (2006) suggest that 90 to 100% of those who drink heavily suffer from alcoholic fatty

1   liver, 10-35% of those evolve to alcoholic steatohepatitis, 8-20% of those evolve to alcoholic
2   cirrhosis, and 1-2% of those develop HCC. Hepatitis C (HCV) infects approximately 170
3   million individuals worldwide with approximately 20% of chronic HCV cases developing liver
4   cirrhosis and 2.5% developing hepatocellular carcinoma (HCC). Infection with hepatitis B
5   (HVB), a non-cytopathic, partially double stranded hepatotropic DNA virus classified as a
6   member of the hepadnaviridae family, is also associated with liver cancer risk with several lines
7   of evidence supporting the direct involvement of HBV in the transformation process (Farazi and
8   DePinho, 2006). El-Serag and Rudolph (2007) suggest that "Epidemiologic research has shown
9   that the great majority of adult-onset HCC cases are sporadic and that many have at lease 1
10 established non-genetic risk factor such as alcohol abuse or chronic HCV or HBV infection.
11 However, most people with these known environmental risk factors never develop cirrhosis or
12 HCC, whereas a sizable minority of HCC case develop among individuals without any known
13 risk factors." "Genetic epidemiology studies in HCC, similar to several other conditions, have
14 fallen short of early expectations that they rapidly and unequivocally would result in
15 identification of genetic variants conveying substantial excess risk of disease and thereby
16 establish the groundwork for effective genetic screening for primary prevention."
17
18 **3.1.4. Issues Associated with Target Cell Identification**
19
20   Another outstanding and important question in HCC pathogenesis involves the cellular
21 origin of this cancer. The liver is made up of a number of cell types showing different
22 phenotypes and levels of differentiation. Which cell types are targets of hepatocarcinogens and
23 are those responsible for human HCC is a matter of intense debate. Studies over the last decade
24 provide evidence of several types of cells in the liver that can repopulate the hepatocyte
25 compartment after a toxic insult. "Indeed, although the existence of a liver stem cell is often
26 debated, most experts agree that progenitor liver cells are activated, in response to significant
27 exposure to hepatotoxins. Also, progenitor cells derived from nonhepatic sources, such as bone
28 marrow and pancreas, have been demonstrated recently to be capable of differentiating into
29 mature hepatocytes under correct microenvironmental conditions (Gandillet et al., 2003). At
30 present, analyses of human HCCs for oval cell markers, comparison of their gene-expression
31 patterns with rat fetal hepatoblasts and the cellular characteristics of HCC from various animal
32 models have provided contrasting results about the cellular origin of HCC and imply dual origins
33 from either oval cells or mature hepatocytes. The failure to identify a clear cell of origin for
34 HCC might stem from the fact that there are multiple cells of origin, perhaps reflecting the
35 developmental plasticity of the hepatocyte lineage. The resolution of the HCC cell of origin
36 issue could affect the development of useful preventative strategies to target nascent neoplasms,
37 foster an understanding of how HCC-relevant genetic lesions function in that specific cell-
38 development context and increase our ability to develop more accurate mouse models in which
39 key genetic events are targeted to the appropriate cellular compartment (Farazi and DePinho,
40 2006). Two reviews by Librecht (2006) and Wu and Chen (2006) provide excellent summaries
41 of the issues involved in identifying the target cell for HCC and the review by Roskams et al
42 (2004) provides a current view of the "oval cell" its location and human equivalent. Recent
43 reports by Best and Coleman (2007) suggest another type of liver cell is also capable of

1 proliferation and differentiating into small hepatocytes (i.e., small hepatocyte-like progenitor
2 cell).
3
4   The review by Librecht (2006) provides and excellent description of the controversy and
5 data supporting different views of the cells of origin for HCC. "In recent years, the results of
6 several studies suggest that human liver tumors can be derived from hepatic progenitor cells
7 rather than from mature cell types. The available data indeed strongly suggest that most
8 combined hepatocellular-cholangiocarcinomas arise from hepatic progenitor cells (HPCs) that
9 retained their potential to differentiate into the hepatocyte and biliary lineages. Hepatic
10 progenitor cells could also be the basis for some hepatocellular carcinomas and hepatocellular
11 adenomas, although it is very difficult to determine the origin of an individual hepatocellular
12 carcinoma. There is currently not enough data to make statements regarding a hepatic progenitor
13 cell origin of cholangiocarcinoma. The presence of hepatic progenitor cell markers and the
14 presence and extent of the cholangiocellular component are factors that are related the prognosis
15 of hepatocellular carcinomas and combined hepatocellular-cholangiocarcinomas, respectively."
16 "The traditional view that adult human liver tumors arise from mature cell types has been
17 challenged in recent decades." "HPCs are small epithelial cells with an oval nucleus, scant
18 cytoplasm and location in the bile ductules and canals of Hering. HPCs can differentiate towards
19 the biliary and hepatocytic lineages. Differentiation towards the biliary lineage occurs via
20 formation of reactive bile ductules, which are anastamosing ductules lined by immature biliary
21 cells with a relatively large and oval nucleus surrounded by a small rim of cytoplasm.
22 Hepatocyte differentiation leads to the formation of intermediate hepatocyte-like cells which are
23 defined as polygonal cells with a size intermediate between than of HPCs and hepatocytes. In
24 most liver diseases, hepatic progenitor cells are "activated" which means that they proliferate and
25 differentiate towards the hepatocytic and/or biliary lineages. The extent of activation is
26 correlated with disease severity." "HPCs and their immediate biliary and hepatocytic progeny
27 not only have a distinct morphology, but they also express several markers, with many also
28 present in bile duct epithelial cells. Immunohistochemistry using antibodies against these
29 markers facilitates the detection of HPCs. The most commonly used markers are cytokeratin
30 (CK) 19 and CK7." "The proposal that a human hepatocellular carcinoma does not necessarily
31 arise from mature hepatocyte, but could have HPC origin, has classically been based on three
32 different observations. Each of them, however, gives only indirect evidence that can be
33 disputed." "Firstly, it has been shown that HPCs are the cells of origin of HCC in some animal
34 models of hepatocarcinogenesis, which has led to the suggestion that this might also be the case
35 in humans. However, in other animal models, the HCCs arise from mature hepatocytes and not
36 from HPCs or reactive bile ductular cells (Bralet et al 2002; Lin et al 1995– DEN treated rats).
37 Since it is currently insufficiently clear which of these animal models accurately mimics human
38 hepatocarcinogenesis, one should be careful about extrapolating data regarding HPC origin of
39 HCC in animal models to the human situation." "Secondly, liver diseases that are characterized
40 by the presence of carcinogens and development of dysplastic lesions also show HPC activation.
41 Therefore, the suggestion has been made that HPCs form a "target population" for carcinogens,
42 but this is only a theoretical possibility not supported by experimental data." "Thirdly, several
43 studies have shown that a considerable proportion of HCCs express one or more HPC markers

1   that are not present in normal mature hepatocytes. Due to the fact that most HPC markers are
2   also expressed in the biliary lineage, the term "biliary marker" has been used in some of these
3   studies. The "maturation arrest" hypothesis states that genetic alterations occurring in a HPC, or
4   its immediate progeny, cause aberrant proliferation and prevent its normal differentiation.
5   Further accumulation of genetic alterations eventually leads to malignant transformation of these
6   incompletely differentiated cells. The resulting HCC expresses HPC markers as evidence of its
7   origin. However, expression of HPC markers can also be interpreted in the setting of the
8   "dedifferentiation" hypothesis, which suggests that the expression of HPC markers is acquired
9   during tumor progression as a consequence of accumulating mutations. For example,
10   experiments in which human HCC cells lines were transplanted into nude mice have nicely
11   shown that the expression of HPC marker, CK19, steadily increased when the tumors became
12   increasingly aggressive and metastasized to the lung, Thus, the expression of CK19 in a HCC
13   does not necessarily mean that the tumor has a HPC origin, but it can also be mutation-induced,
14   acquired expression associated with tumor progression. Both possibilities are not mutually
15   exclusive." "For an individual HCC that expresses a HPC marker, it remains impossible to
16   determine whether this marker reflects the cellular origin and/or is caused by tumor progression.
17   This can only be elucidated by determining whether HCC contains cells that are ultrastructurally
18   identical to HPCs in non-tumor liver.
19
20   Similarly, the review by Wu and Chen (2006) also presents a valuable analysis of these
21   issues and state the "The question of whether hepatocellular carcinomas arises from the
22   differentiation block of stem cells or dedifferentiation of mature cells remains controversial.
23   Cellular events during hepatocarcinogenesis illustrate that HCC may arise for cells at various
24   stages of differentiation in the hepatic stem cell lineage." "The role of cancer stem cells has been
25   demonstrated for some cancers, such as cancer of the hematopoietic system, breast and brain.
26   The clear similarities between normal stem cell and cancer stem cell genetic programs are the
27   basis of the a proposal that some cancer stem cells could derived form human adult stem cells.
28   Adult mesenchymal stem cells (MSC) may be targets for malignant transformation and undergo
29   spontaneous transformation following long-term in vitro culture, supporting the hypothesis of
30   cancer stem cell origin. Stem cells are not only units of biological organization, responsible for
31   the development and the regeneration of tissue and organ systems, but are also targets of
32   carcinogenesis. However, the origin of the cancer stem cell remains elusive." "Three levels of
33   cells that can respond to liver tissue renewal or damage have been proved (1) mature liver cells,
34   as "unipotential stem cells", which proliferate under normal liver tissue renewal and respond
35   rapidly to liver injury, (2) oval cells, as bipotential stem cells, which are activated to proliferate
36   when the liver damage is extensive and chronic or if proliferation of hepatocytes is inhibited; and
37   (3) bone marrow stem cells, as multipotent liver stem cells, which have a very long proliferation
38   potential. There are two major nonexclusive hypotheses of the cellular origin of cancer; from
39   stem cells due to maturation arrest or from dedifferentiation of mature cells. Research on hepatic
40   stem cells in hepatocarcinogenesis has entered a new era of controversy, excitement and great
41   expectations." "The two major hypotheses about the cellular origination of HCC have been
42   discussed for almost 20 years. Debate has centered on whether or not HCC originates from the
43   differentiation block of stem cells or dedifferentiation of mature cells. Recent research suggests

1   that HCC may originate from the transdifferentiation of bone marrow cells. In fact, there might
2   be more than one type of carcinogen target cell. The argument about the origination of HCC
3   becomes much clearer when viewed from this viewpoint: poorly differentiated HCC orginate
4   from bone marrow stem cells and oval cells, while well-differentiated HCC originates form
5   mature hepatocytes." "The cellular events during hepatocarcingenesis illustrate that HCC may
6   arise from cells at various stages of differentiation in the hepatocyte lineage. There are four
7   levels of cells in the hepatic stem cell lineage: bone marrow cell, hepato-pancreas stem cell, oval
8   cell and hepatocyte. HSC and the liver are known to have a close relationship in early
9   development. Bone marrow stem cells could differentiate into oval cells, which could
10 differentiate into heptaocytes and duct cells. The development of pancreatic and liver buds in
11 embryogenesis suggests the existence of a common progenitor cells to both the pancreas and
12 liver. All of the four levels of cells in the stem cell lineage may be targets of
13 hepatocarcinogenesis."
14
15   Along with the cell types described as possible targets and participants in HCC, Best and
16 Coleman (2007) describe yet another type of cell in the liver that can respond to hepatocellular
17 injury which they term small hepatocyte-like progenitor cells and conclude that they are not the
18 progeny of oval cells, but represent a distinct liver progenitor cell population. Another potential
19 regenerative cell is the small hepatocyte-like progenitor cell (SHPC). SHPCs share some
20 phenotypes with hepatocytes, fetal hepatoblasts, and oval cells, but are phenotypically distinct.
21 They express markers such as albumin, transferring, and AFP and possess bile canaliculi and
22 store glycogen.
23
24   A recent review by Roskams et al. (2004) provides a current view of the "oval cell" its
25 location and human equivalent. They conclude that "while similarities exist between the
26 progenitor cell compartment of human and rodent livers, the different rodent models are not
27 entirely comparable with the human situation, and use of the same term has created confusion as
28 to what characteristics may be expected in the human ductular reaction. For example, a defining
29 feature of oval cells in many rodent models of injury is production of alpha-fetoprotein, whereas
30 ductular reactions in humans rarely display such expression. Therefore we suggest that the "oval
31 cell" and "oval –like cell" no longer be used in description of human liver."
32
33   In the chronic hepatitis and cancer model of Vig et al. (2006) it is not the oval cells or
34 SHPCs that are proliferating but the mature hepatocytes, thus supporting theories that it is not
35 only oval cells that are causing proliferations leading to cancer. Vig et al., (2006) also report that
36 studies in mice an humans indicate that oval cells also may give rise to liver tumors and that oval
37 cells commonly surround and penetrate human liver tumors, including those caused by hepatitis
38 B. Tarsetti et al. (1993) suggest that although some studies have suggested that oval cells are
39 directly involved in the formation of HCC others assert that HCC originates from preneoplastic
40 foci and nodules derived from hepatocytes and report that HCC evolved in their model of liver
41 damage from hepatocytes, presumably hepatocellular nodules, and not from oval cells. They
42 also suggest that proliferation alone may not lead to cancer. Recent studies that follow the
43 progression of hepatocellular nodules to HCC in humans (see Section 3.2.4 below) suggest an

1 evolution from nodule to tumor.
2
3 **3.1.5. Status of Mechanism of Action for Human HCC**
4
5   The underlying molecular mechanisms leading to hepatocarcinogeneis remain largely
6 unclear (Yeh et al., 2007).   Although HCC is multistep, and its appearance in children suggest a
7 genetic predisposition exists, the inability to identify most of the predisposing genes and how
8 their altered expression relates to histological lesions that are the direct precursors to HCC, has
9 made it difficult to identify the rate limiting steps in hepatocarcingenesis (Feitelson et al., 2002).
10 Calvisi et al. (2007) report that although the major etiological agents have been identified, the
11 molecular pathogenesis of HCC remains unclear and that while deregulation of a number of
12 oncogenes (e.g., *c-Myc*, *cyclin D1* and *β-catenin* and tumor suppressor genes including *$P16^{INK4A}$*,
13 *P53*, *E-cadherin*, *DLC-1*, and *pRb*) have been observed at different frequencies in HCC, the
14 specific genes and the molecular pathways that play pivital roles in liver tumor development
15 have not been identified. Indeed rather than simple patterns of mutations, pathways that are
16 common to cancer have been identified through study of tumors and through transgenic mouse
17 models. Branda and Wands (2006) state that the molecular factors and interactions involved in
18 hepatocarcinogenesis are still poorly understood but are particularly true with respect to genomic
19 mutations, "as it has been difficult to identify common genetic changes in more than 20% to 30%
20 of tumors." As well as phenotypically heterogeneous, "it is becoming clear that HCCs are
21 genetically heterogeneous tumors." The descriptions of heterogeneity of tumors and of pathway
22 disruptions common to cancer are also shown for liver tumors (see Sections 3.1.6, and 3.1.8,
23 below). However, many of these studies focus on the end process and of examination of the
24 genomic phenotype of the tumor for inferences regarding clinical course, aggressiveness of
25 tumor, and consistency with other forms of cancer. As stated above, the events that produce
26 these tumors from patients with conditions that put them at risk,are not known.
27
28   El-Serag and Rudolph (2007) suggest that risk of HCC increases with at the cirrhosis
29 stage when liver cell proliferation is decreased and that acceleration of carcinogenesis at this
30 stage may result from telomere shortening (resulting in limitations of regenerative reserve and
31 induction of chromosomal instability), impaired hepatocyte proliferation (resulting in cancer
32 induction by loss of replicative competition), and altered milieu conditions that promote tumor
33 cell proliferation. "When telomeres reach a critically short length, chromosome uncapping
34 induces DNA damage signals, cell-cycle arrest, senescence, or apoptosis. Telomeres are
35 critically short in human HCC and on the single cell level telomere shortening correlated with
36 increasing aneuploidy in human HCC." "Chemicals inhibiting hepatocyte proliferation
37 accelerate carcinogen-induced liver tumor formation in rats as well as the expansion and
38 transformation of transplanted hepatocytes. It is conceivable that abnormally proliferating
39 hepatocytes would not expand in healthy regenerating liver but would expand quickly and
40 eventually transform in the growth restrained cirrhotic liver". "Liver mass is controlled by
41 growth factors – mass loss through could provide a growth stimulatory macroenviroment. For
42 the microenvironment, cirrhosis activates stellate cells resulting in increased production of
43 extracellular matrix proteins, cytokines, growth factors, and products of oxidative stress."

1   Like other cancers, genomic instability is a common feature of human HCC with various
2   mechanisms thought to contribute, including telomere erosion, chromosome segregation defects,
3   and alteration in DNA damage-response pathways. In addition to genetic events associated with
4   the development of HCC (p53 inactivation, mutation in β-catenin, overexpression of ErbB
5   receptor family members, and overexpression of the MET receptor whose ligand is HGF) various
6   cancer-relevant genes seem to be targeted on the epigenetic level (methylation) in human HCC
7   (Farazi and DePinho, 2006). Changes in methylation have been detected in the earliest stages of
8   hepatocarcinogenesis and to a greater extent in tumour progression (Lee et al., 2003). Seitz and
9   Stickel (2006) report that aberrant DNA hypermethylation (a silencing effect on genes) may be
10   associated with genetic instability as determined by the loss of heterozygosity and microsatellite
11   instability in human HCC due to chronic viral hepatitis and that modifications of the degree of
12   hepatic DNA methylation have also been observed in experimental models of chronic
13   alcoholism. Farazi and DePinho (2006) report that two of the key molecules that involved in
14   DNA damage response, p53 and BRCA2, seem to have roles in destabilizing the HCC genome
15   (Collin 2005). The inactivation of p53 through mutation or viral oncoprotein sequestration is a
16   common event in HCC and p53 knock in mouse models containing dominant point mutations
17   have been shown to cause genomic instability. However, Farazi and DePinho (2006) note that
18   despite documentation of deletions or mutations in these and other DNA damage network genes,
19   their direct roles in the genomic instability of HCC have yet to be established in many genetic
20   model systems.
21
22   Telomere shortening has been described as a key feature of chronic hyperproliferative
23   liver disease (Urabe et al 1996; Miura et al 1997; Rudolf et al 2001: Kitada et al 1995),
24   specifically occurring in the hepatocyte compartment. These observations have fueled
25   speculation that telomere shortening associated with chronic liver disease and hepatocyte
26   turnover contribute to the induction of genomic instability that drives human HCC (Farazi and
27   DePinho, 2006). Defects in chromosome segregation during mitosis result in aneuploidy, a
28   common cytogenetic feature of cancer cell including HCC (Farazi and DePinho, 2006).
29
30   Several studies have attempted to categorize genomic changes in relation to tumour state.
31   In general, high levels of chromosomal instability seem to correlate with the de-differentiation
32   and progression of HCC (Wilkens et al., 2004). Several studies have suggested certain
33   chromosomal changes to be specific to dysplastic lesions, early –stage and late-stage HCCs, and
34   metastases. It is important to note that the studies that have attempted to compare genomic
35   profiles and tumour state are few in number, often did not classify HCCs on the basis of etiology,
36   and used relatively low-resolution genome-scanning platforms (Farazi and DePinho, 2006).
37   Farazi and DePinho (2006) note that it should be emphasized that although genome –etiology
38   correlates reported in some studies, are intriguing, several studies have failed to uncover
39   significant differences in genomic changes between different etiological groups, although the
40   outcome might related to small sample sizes and the low-resolution genome –scanning platform
41   used.

3.1.6. Pathway and Genetic Disruption Associated with HCC and Relationship to Other Forms of Neoplasia

In their landmark paper, Hanahan and Weinberg (2000) suggested that the vast catalog of cancer cell genotypes were a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth; self-sufficiency in growth signals, insensitivity to growth –inhibitory (antigrowth signals), elevation of programmed cell death (apoptosis), limitless replication potential, sustained angiogenesis, and tissue invasion and metastasis. They proposed that these six capabilities are shared in common by most and perhaps all types of human tumors and, while virtually all cancers must acquire the same six hallmark capabilities, their means of doing so would vary significantly, both mechanistically and chronologically. It was predicted that in some tumors, a particular genetic lesions may confer several capabilities simultaneously, decreasing the number of distinct mutational steps required to complete tumorigenesis. Loss of the p53 tumor suppressor was cited as an example that could facilitate both angiogenesis and resistance to apoptosis and to enable the characteristic of genomic instability. The paths that cells could take on their way to becoming malignant were predicted to be highly variable, and within a give cancer type, mutation of a particular target genes such as *ras* or *p53* could be found only in a subset of otherwise histologically identical tumors. Furthermore, mutations in certain oncogenes and tumor suppressor genes could occur early in some tumor progression pathways and late in others. Genes known to be functionally altered in "cancer" were identified as including Fas,Bcl2, Decoy R, Bax, Smads, TFGβR, p15, p16, Cycl D, Rb, HPV E7, ARF, PTEN, Myc, Fos, Jun, Ras, Abl, NF1, RTK, TGFα, Integrins, E-cadherin, Src, β-catenin, APC, and WNT.

Branda and Wands (2006) report that two signal transduction cascades that appear to be very important are insulin/IFG-1/IRS-1/MAPK and Wnt/Frizzled/ β-catenin pathways which are activated in over 90% of HCC tumors (Branda and Wands, 2006). Feitelson et al. (2002) reported that "In addition to NF-κB, up-regulated expression of rhoB has been reported in some HCCs. RhoB is in the *ras* gene family, is associated with cell transformation, and may be a common denominator to both viral and non-viral hepatocarcingenesis. Activation of ras and NF-κB, combined with down regulation of multiple negative growth regulatory pathways, then, may contribute importantly to early steps in hepatocarcinogenesis. Thus viral proteins may alter the patterns of hepatocellular gene expression by transcriptional trans-regulation." "Another early event appears to involve the mutation of β-catenin, which is a component of the Wnt signal transduction pathway whose target genes include c-myc, c-jun, cyclin D1, fibronectin, the connective tissue growth factor WISP, and matrix metaolloproteinases." Boyault et al (2007) report that "altogether, the principle carcinogenic pathways known to be deregulated in HCC are inactivation of TP53, Wnt/wingless activation mainly through CTNNB1 mutations activating β-catenin- and AXIN1-inactivating mutations, retinoblastoma inactivation through RB1 and CDKN2A promoter methylation and rare gene mutations, insulin growth factor activation through IGF2 overexpression, and IGF2R-inactiving mutations."

1   El-Serag and Rudolph suggest that "in general, the activation of oncogenic pathways in
2   human HCC appears to be more heterogeneous compared with other cancer types." El-Serag
3   and Rudolph (2007) report that the p53 pathway is a major tumor-suppressor pathway that (1)
4   limits cell survival and proliferation (replicative senescence) in response to telomere shortening
5   (2) induces cell-cycle arrest in response to oncogene activation (oncogene-induced senescence),
6   (3) protects genome integrity, and (4) is affected at multiple levels in human HCC. "p53
7   mutations occur in aflatoxin induced HCC (>50%) and with lower frequency (20-40%) in HCC
8   not associated with aflatoxin."   In addition, "the vast majority of human HCC overexpresses
9   gankyrin, which inhibits both Rb checkpoint and p53 checkpoint function." "The p16/Rb
10   checkpoint is another major pathway limiting cell proliferation in response to telomere
11   shortening, DNA damage, and oncogene activation. In human HCC the Rb pathway is disrupted
12   in more than 80% of cases, with repression of p16 by promoter methylation being the most
13   frequent alteration. Moreover, expression of gankyrin (an inhibitor of p53 and Rb checkpoint
14   function) is increased in the vast majority of human HCCs, indicating that the Rb checkpoint is
15   dysfunctional in the vast majority of human HCCs." "The frequent inactivation of p53 in human
16   HCC indicates that abrogation of p53-dependent apoptosis could promote hepatocarcinogenesis.
17   The role of impairment of p53-independent apoptosis for hepatocarcinogenesis remains to be
18   defined." "Activation of the β-catenin pathway frequently occurs in mouse and human HCC
19   involving somatic mutations, as well as transcriptional repression of negative regulators. An
20   activation of the Akt signaling and impaired expression of phosphatase and tensin homolog
21   (PTEN) (a negative regulator of Akt) have been reported in 40-60% of Human HCC." They
22   suggest that although *Myc* is a potent oncongene inducing hepatocarcinogenesis in mouse models
23   the data on human HCC are heterogeneous and further studies are required.
24
25   **3.1.7. Epigenetic Alterations in HCC**
26
27   The molecular pathogenesis of HCC remains largely unknown but it is presumed that the
28   development and progression of HCC are the consequence of cumulative genetic and epigenetic
29   events similar to those described in other solid tumors (Calvisi et al., 2006). Calvisi et al (2007)
30   provide a good summary of DNA methylation status and cancer as well as it's status in regard to
31   HCC:
32   Aberrant DNA methylation occurs commonly in human cancers in the forms of genome-
33   wide hypomethylation and regional hypermethylation. Global DNA hypomethylation
34   (also known as demethylation) is associated with activation of protooncogenes, such as c-
35   Jun, c-Myc, and c-HA-Ras, and generation of genomic instability. Hypermethylation on
36   CpG islands located in the promoter regions of tumor suppressor genes results in
37   transcriptional silencing and genomic instability. CpG hypermethylation (also known as
38   de novo methylation) acts as an alternative and/or complementary mechanisms to gene
39   mutations causing gene inactivation, and it is now recognized as an important mechanism
40   in carcinogenesis. Although the mechanism(s) responsible for de novo methylation in
41   cancer are poorly understood, it has been hypothesized that epigenetic silencing depends
42   on activation of a number of proteins known as DNA methyltransferases (DNMTs) that
43   posses de novo methylation activity. The importance of DNMTs in CpG methylation

1   was substantiated by the observation that genetic disruption of both DNMT1 and
2   DNMT3b genes in HCT116 cell lines nearly eliminated methyltransferase activity.
3   However, more recent findings indicate that the HCT116 cells retain a truncated,
4   biologically active form of DNMT1 and maintain 80% of their genomic methylation.
5   Further reduction of DNMT1 levels by an siRNA approach resulted in decreased cell
6   viability, increased apoptosis, enhanced genomic instability, checkpoint defects, and
7   abrogation of replicative capacity. These data show that DNTM1 is required for cell
8   survival and suggest that DNTM1 has additional functions that are independent of its
9   methyltransferase activity. Concomitant overexpression of DNMT1, -3A, and -3b has
10 been found in various tumors including HCC. However, no changes in the expression of
11 DNMTs were found in other neoplasms, such as colorectal cancer, suggesting the
12 existence of alternative mechanisms. In HCC, a novel DNMT3b splice variant, known as
13 DNMT3b4 is overexpressed. DNMT3b4 lacks DNMT activity and competes with
14 DNMT2b3 for targeting of pericentromeric satellite regions in HCC, resulting in DNA
15 hypomethylation of these regions and induction of chromosomal instability, further
16 linking aberrant methylation and generation of genomic alterations.

17 It is now well accepted that methylation changes occur early and ubiquitously in cancer
18 development. The case has been made that tumor cell heterogeneity is due, in part, to
19 epigenetic variation in progenitor cells and that epigenetic plasticity together with genetic
20 lesions drive tumor progression (Feinberg et al., 2006).

21 A growing number of genes undergoing aberrant CpG island hypermethylation in HCC
22 have been discovered, suggesting that de novo methylation is an important mechanism
23 underlying malignant transformation in the liver. However, most of the previous studies
24 have focused on a single or a limited number of genes, and few have attempted to analyze
25 the methylation status of multiple genes in HCC and associated chronic liver diseases. In
26 addition, the functional consequence(s) of global DNA hypomethylation and CpG island
27 hypermethylation in human liver cancer has not been investigated to date. Furthermore,
28 to our knowledge no comprehensive analysis of CpG island hypermethylation involving
29 activation of signaling pathways has been performed.

30 Calvisi et al. (2007) report that global gene expression profiles show human HCC to
31 harbor common molecular features that differ greatly from those of non-tumorous surrounding
32 tissues, and that human HCC can be subdivided into 2 broad but distinct subclasses that are
33 associated with length of patient survival. They further suggest that aberrant methylation is a
34 major event in both early and late stages of liver malignant transformation and might constitute a
35 critical target for cancer risk assessment, treatment, and chemoprevention of HCC. Calvisi et al
36 (2007) conducted analysis of methylation status of genes selected based on their capacity to
37 modulate signaling pathways (*Ras*, Jak/Stat, Wingless/Wnt, and RELN) and/or biologic features
38 of the tumors (proliferation, apoptosis, angiogenesis, invasion, DNA repair, immune response,
39 and detoxification). Normal livers were reported to show the absence of promoter methylation
40 for all genes examined. At least 1 of the genes involved in inhibition of Ras (*ARH1, CLU,*
41 *DAB2, hDAB21P, HIN-1, HRASL, LOX, NORE1A, PAR4, RASSF1A, RASSF2, RASSF3,*
42 *RASSF4, RIG, RRP22, and SPRY2 and -4*), Jak/Stat (*ARH1,CIS, SHP1, PIAS-1, PIAS-γ, SOCS1,*

-2, and -3, SYK, and GRIM-19), and Wnt/β-catenin (*APC, E-cadherin, γ-catenin, SFRP1, -2, -4, and -5, DKK-1 and -3, WIF-1 and HDPR1*) pathways was affected by de novo methylation in all HCC. A number of these genes were also reported to be highly methylated in the surrounding non-tumorous liver. In contrast, inactivation of at least 1 of these genes implicated in the RELN pathway (DAB1, reelin) was detected differentially in HCC of subclasses of tumor that had difference in tumor aggressiveness and progression. Epigenetic silencing of multiple tumor suppressor genes maintains activation of the *Ras* pathway with a major finding in the Calvisi et al (2007) study to be the concurrent hypermethylation of multiple inhibitors of the *Ras* pathway with *Ras* was significantly more active in HCC than in surrounding or normal livers. Also important, was the finding of no significant association between methylation patterns and specific etiologic agents (i.e., HVB, HVC, ethanol, etc) was detected further substantiating the conclusion that aberrant methylation is a ubiquitous phenomenon in hepatocarcingenesis. "Current evidence suggests that hypomethylation might promote malignant transformation via multiple mechanisms, including chromosome instability, activation of protooncogenes, reactivation of transposable elements, and loss of imprinting." "The degree of DNA hypomethylation progressively increased from non-neoplastic livers to fully malignant HCC, indicating that genomic hypomethylation is an important prognostic factors in HCC, as reported for brain, breast, and ovarian cancer." Calvisi et al. (2007) also report that regional CpG hypermethylation was also enhanced during the course of HCC disease and that the study of tumor suppressor gene promoters showed that CpG methylation was frequently detected both in surrounding non-tumorous livers and HCC.

3.1.8. Heterogeneity of Preneoplastic and HCC Phenotypes

A very important question for the treatment of HCC in humans is early detection. Research has focused on identification of lesions that will progress to HCC and to also determine from the phenotype of the nodule and genetic expression its cell source, likely survival, and associations with etiologies and MOAs. As with rodent models where preneoplastic foci have been observed to be associated with progression to adenoma and carcinoma, nodules observed in humans with high risk for HCC have been observed to progress to HCC. In humans histomorphology of HCC is notoriously heterogeneous (Yeh et al., 2007). Although much progress has been made, there is currently not universally accepted staging system for HCC partly because of the natural course of early HCC is unknown and the natural progression of intermediated and advanced HCC are quite heterogeneous (Thorgeirsson, 2006). Nodules are heterogeneous as well with differences in potential to progress to HCC. Chen et al (2002) report that standard clinical pathological classification of HCC has limited valued in predicting the outcome of treatment as the phenotypic diversity of cancer is accompanied by a corresponding diversity in gene expression patterns. There is also histopathological variability in the presentation of HCC in geographically diverse regions of the world with some slow growing, differentiated HCC nodules surrounded by a fibrous capsule are common among Japanese but, in contrast, a"febrile" form of HCC, characterized by leukocytosis, fever, and necrosis within a poorly differentiated tumor to be common in South African blacks (Feitelson et al., 2002).

1   A multistep process is suggested histologically, where HCC appears within the context of
2   chronic hepatitis and/or cirrhosis within regions of the liver cell dsyplasia or adenomatous
3   hyperplasia (Feitelson et al., 2002). Kobayashi et al. (2006) report that the higher the grade of
4   the nodule the higher the percentage that will progress to HCC with 18.8% of all nodules and
5   regenerative lesions going on to become HCC, 53.3% remaining unchanged, and 27.9%
6   disappearing in the observation period of 0.1 to 8.9 years. Borzio et al (2003) report that the rate
7   of liver malignant transformation was 40% in larger regenerative nodules, low-grade dysplastic,
8   and high-grade dysplastic nodules with higher grade of dysplasia extranodular detection of large
9   cell change and hyperchroic pattern associated with progression to HCC. Yeh et al (2007) report
10 that nuclear staining for Ki-67 and Topo II-α (a nuclear protein targeted by several
11 chemotherapeutic agents) significantly increased in the progression from cirrhosis, through high
12 grade dysplastic nodules to HCC whereas the scores for TGF-α in these lesions showed an
13 inverse relationship. "In comparison with 18 HCC arising in noncirrhotic livers, the expression
14 of TGF-α is significantly stronger in cirrhotic liver than in noncirrhotic parenchyma and its
15 expression is also stronger in HCC arising in cirrhosis than in HCC arising in noncirrhotic
16 patients." They concluded that initiation in cirrhotic and noncirrhotic liver may have different
17 pathways with Transforming growth factor-α (a mitogen activated the EFGR) playing a relative
18 more important role in HCC from cirrhotic liver. Over expression of TGF-α in the liver of
19 transgenic mice induced increased proliferation, dysplasia, adenoma and carcinoma. Yeh et al.
20 (2007) concluded that such high-grade dysplastic nodules are precursor lesions in
21 hepatocarcingenesis and that TGF-α may play an important role in the early events of liver
22 carcinogenesis.
23
24   Moinzadeh et al. (2005) reported in a meta-analysis of all available (n = 785 HCCs) that
25 gains and losses of chromosomal material were most prevalent in a number of chromosomes and
26 that amplifications and deletions occurred on chromosomal arms in which oncogenes (e.g. MYC
27 and 8q24) and tumor suppressor genes (e.g., RB1 on 13q14) are located as well a modulators of
28 the WNT-signaling pathway. However, in multifocal HCC, nodules arising de novo within a
29 single liver have a different spectrum of genetic lesions. "Hence, there are likely to be many
30 paths to hepatocellular carcinoma, and this is why it has been difficult to assign specific
31 molecular alterations to changes in hepatocellular phenotype, clinical, or histopathological
32 changes that accompany tumor development" (Feitelson et al., 2002).
33
34   Serum alpha-fetoprotein (AFP) is commonly used as tumor marker for HCC. Several
35 reports have linked HCC to cytokines in an attempt to find more specific markers of HCC. Jia et
36 al (2007) report that AFP marker allows for identification of a small set of HCC patients with
37 smaller tumors, and these patients have a relatively long-term survival rate following curative
38 treatment. "Presently the only approach to screen for the presence of HCC in high-risk
39 populations is the combination of serum AFP and ultrasonagraphy. However, elevated AFP is
40 only observed in about 60 to 7-% of HCC patients and to a lesser extent (33-65%) in patients
41 with smaller HCCs.   Moreover, nonspecific elevation of serum AFP has been found in 15% to
42 58% of patients with chronic hepatitis and 11% to 47% of patients with liver cirrhosis." Soresi et
43 al (2006) report that Serum IL-6 levels are low in physiological conditions, but increase

1 considerably pathological conditions such as trauma, inflammation and neoplasia. In tumors IL-
2 6 may be involved in promoting the differentiation and growth of target cells. "Many works have
3 reported high serum IL-6 levels in various lifer diseases such as acute hepatitis, primary biliary
4 cirrhosis, chronic hepatitis (hepatitis C) and HCV-correlated liver cirrhosis and in hepatocellular
5 carcinoma." Soresi et al (2006) report that patients with HCC group had higher IL-6 values than
6 those with cirrhosis and that "higher-staged" patients had the highest IL-6 levels. Hsia et al
7 (2007) also examined IL-6, IL-10 and hepatocyte growth factor (HGF) as potential markers for
8 HCC. "The expression of IL-6 or IL-10 or higher level of HGF or AFP was observed only 0-3%
9 of normal subjects. Patients with HCC more frequently had higher IL-6 and IL-10 levels, where
10 as HGF levels in HCC patients were not significantly elevated compared to patients with chronic
11 hepatitis or non-HCC tumors (but greater than controls). Among patients with low AFP level,
12 IL-6 or IL-10 expression was significantly associated with the existence of HCC. Patients with
13 large HCC (>5 cm) more often had increased IL-6, IL-10 or AFP levels. Serum levels of IL-6
14 and IL-10 are frequently elevated in patients with HCC but not in benign liver disease or non-
15 HCC tumors."
16
17   Nuclear DNA content and ploidy have also been the subjects of several studies through
18 the years for identification of pathways for prediction of survival or origin of tumors. Nakajima
19 et al. (2004) report that p53 loss can contribute to the propagation of damaged DNA in daughter
20 cells through the inability to prevent the transmission of inaccurate genetic material, considered
21 to be one of the major mechanisms for the emergence of aneuploidy in tumors with inactivated
22 p53 protein and the increasing ploidy in HCC was associated with disturbance in p53. McEntee
23 et al. (1991) reported that specimens from 74 patients who underwent curative resection for
24 primary HCC and analyzed for DNA content, [i.e., tumors were classified as DNA aneulploid if
25 a separate peak was present from its standard large diploid peak (2C) and tetaploid peak (4C)]
26 were 33% were DNA diploid, 30% were DNA tetraploid/ polyploidy, and 37% were aneuploid
27 of the primary tumors examined. Non-tumor controls were diploid and survival was not
28 different between patients with diploid vs. nondiploid tumors. Zeppa et al (1998) reported ploidy
29 in 84 hepatocellular carcinomas diagnosed by fine-needle aspiration biopsy to have 68 cases
30 were aneuploid and 16 euploid (9 diploid and 7 polyploid) with median survival of 38 months for
31 patients with diploid HCC and 13 months for aneuploid HCC. Lin et al (2003) report in their
32 study of fine needle aspiration of HCC that "the ratio of S and G2/M periods of DNA, which
33 reflect cell hyperproliferation, in the group with HCC tumors> 3cm in diameter were markedly
34 higher than those of the group with nodules< 3 cm in diameter and the group with hyperplastic
35 nodules." "DNA analysis of aspiration biopsy tissues acquired from intrahepatic benign
36 hyperplastic nodules showed steady diploid (2c) peak that stayed in G1 period. DNA analysis of
37 aspiration biopsy tissues acquired from HCC nodules showed S period of hyperproliferation and
38 G2/M period. The DNA analysis of HCC nodules showed aneuploid peak." They concluded that
39 the biological behavior of the cell itself that the normal tissue, reactive tissue and benign tumor
40 all have normal diploid DNA but like most other malignant tumors, HCC appears to have
41 polyploid DNA, especially aneuploid DNA." Attallah et al. (1999) report small needle liver
42 biopsy data to show HHC to be 21.4% diploid, 50% aneuploid and 28.6% tetraploid and that
43 higher ploidies (aneuploid and tetraploid) were observed in human liver cancer than residual

1   tissues, although in some cases there was increased aneuploidy (cirrhosis, 37%, hepatitis ~ 50%).
2   Of note for the study is the lack of appropriate control tissue and uncertainty as to how some of
3   their diploid cells could have been binucleate tetraploid cells. Anti et al, (1994) reported
4   reduction in binuclearity in the chronic hepatitis and cirrhosis groups that was significantly
5   correlated with a rise in the diploid/polyploidy ratio and that precancerous and cancerous nodules
6   within cirrhotic liver show an increased tendency toward diploidy or the emergence of aneuploid
7   populations. They note that a number of investigators have noted significantly increased
8   hepatocyte diploidization during the early stages of chemically induced carcinogenesis in rat
9   liver, but other experimental findings indicate that malignant transformation can occur after any
10 type of alteration in ploidy distribution. On the other hand, Melchiorri et al (1994) note that
11 several studies using flow cytometric or image cytometric methods reported high DNA ploidy
12 values in 50-77% of the examined HCCs and that the presence of aneuploidy was significantly
13 related to a poor patient prognosis. They report that the DNA content of mononucleated and
14 binucleated hepatocytes obtained by ultrasound-guided biopsies of 10 macroregenerative nodules
15 without histologic signs of atypia the lesions with the greater fraction of fraction of
16 mononucleated hepatocytes were diagnosed as HCCs during the clinical follow-up with results
17 also suggesting that diploid and tetraploid stem cell lines are the main lines of the HCCs as well
18 as a reduction in the percentage of binucleated hepatocytes in HCC. Gramantieri et al (1996)
19 report that the percentage of binucleated cells was reduced in most of HCC they studied (i.e., the
20 mean percentage of binucleated cells 9% in comparison to 24% found in normal liver) and that
21 most HCC, as many other solid neoplasms, showed altered nuclear parameters.
22
23   Along with reporting pathways that are perturbed in HCC, emerging evidence also shows
24 that signatures of pathway are predictive of clinical characteristics of HCC. A number of studies
25 have examined gene expression in tumors to try to determine which pathways may have been
26 disturbed in an attempt to predict survival and treatment options for the patients and to
27 investigate possible MOAs for the tumor induction and progression. Chen et al. (2002)
28 described a systematic characterization of gene expression patterns in human liver cancers using
29 cDNA microarrays to study tumor and non-tumor liver tissues in HCC patients and of note did
30 quality assurance on their microarray chips (many studies do not report that they have done so)
31 and examined the effects of hepatitis virus on its subject and identified people with it. Most
32 importantly, Chen et al (2002) provided phenotypic anchoring of each tumor with its genetic
33 profile rather than pooling data. The hierarchical analysis demonstrated that clinical samples
34 could be divided into two major clusters, one representing HCC samples and the other with a few
35 exceptions, representing non-tumor liver tissues. Most importantly, expression patterns varied
36 significantly among the HCC and non-tumor liver samples and that samples from HBV-infected,
37 hepatitis C virus infected, and noninfected individuals were interspersed in the HCC branch.
38 Thus, tumors from people infected with HVB, HVC and noninfected people with HCC were
39 interspersed in the HCC pattern and could be discerned based on etiology. One cluster of genes
40 was highly expressed in HCC samples compared with non-tumor liver tissues included a
41 "proliferation cluster" comprised of genes whose functions are required for cell cycle
42 progression and whose expression levels correlate with cellular proliferation rates with most of
43 the genes in this cluster are specifically expressed in the G2/M phase. Gene profiles for HCC

1 were consistent with fewer molecular features of differentiated normal hepatocytes. Chen et al
2 (2002) noted that both normal and liver tumors are complex tissue compose of diverse cells and
3 that distinct patterns of gene expression seemed to provide molecular signatures of several
4 specific cell types including expression of two clusters of genes associated with T and B
5 lymphocytes, presumably reflecting lymphocytic infiltration into liver tissues, and genes
6 associated with stellate cell activation.   This important finding acknowledges that HCC are not
7 only heterogeneous in hepatocyte phenotype but are made up of many other nonparenchymal cell
8 types and that gene expression patterns reflect that heterogeneity. A gene cluster was also
9 identified at a higher level in HCC that included several genes typically expressed in endothelial
10 cells, including CD34, which is expressed in endothelial cells in veins and arteries but not in the
11 endothelial cells of the sinusoids in non-tumor liver and which mar reflect disruption of the
12 molecular program that normally regulate blood vessel morphogenesis in the liver.
13
14   Of great importance was the investigation by Chen et al. (2002) of whether samples from
15 multiple sites in a single HCC tumor, or multiple separate tumor nodules in one patient, would
16 share a recognizable gene expression signature. With a few instructive exceptions, all the tumor
17 samples from each patient clustered were reported to cluster together. To further examine the
18 relationship among multiple tumor samples from individual patients, we calculated the pairwise
19 comparison for all pairs of samples and samples some primary tumors multiple times. Tumor
20 patterns of gene expression were more highly correlated those seen in samples from the same
21 patient than other patient but every tumor had a distinctive and characteristic gene expression
22 pattern, recognizable in all samples taken from different areas of the same tumor. For multiple
23 discrete tumor masses obtained from six patients, three of these patients had multiple tumors
24 with a shared distinctive gene expression pattern but in three other patients, expression patterns
25 varied between tumor nodules and the difference provided new insights into the sources of
26 variation in molecular and biological characteristics of cancers. Thus, in some patients multiple
27 tumors were from the same clone, as demonstrated by a similar gene expression profile, but for
28 some patients multiple tumors were arising from differing clones within the same liver. In
29 regard to whether the distinctive expression patterns characteristic of each tumor reflect the
30 individuality of the tumor, or are they determined by the patient in whom the tumor arose?,
31 analysis of the expression patterns observed in the two tumor nodules from one patient showed
32 that the two tumors were not more similar than those of an arbitrary pair of tumors from different
33 patients. These results show the heterogeneity of HCC and that "one gene pattern" will not be
34 characteristic of the disease.
35
36   However, HCC did have a pattern that differed from other cancers. Chen et al (2002)
37 analyzed the expression patterns of 10 randomly selected HCC samples and 10 liver metastases
38 of other cancers and reported that the HCC samples and the metastatic cancers clustered into two
39 distinct groups, based on difference in their patterns of gene expression. Although some of the
40 HCC samples were poorly differentiated and expressed the genes of the liver-specific cluster at
41 very low levels compared to with either normal liver or well-differentiated HCC, the genes of the
42 liver-specific cluster were reported to be consistently expressed at higher levels in HCC than in
43 tumors of nonliver origin with metastatic cancers originating from the same tissue typically

1   clustered together, expressing genes characteristic of the cell types of origin. Thus, liver cancer
2   was distinguishable from other cancer even though very variable in expression and
3   differentiation state.
4
5   In attempt to create molecular prognostic indices that can be used for identification of
6   distinct subclasses of HCC that could predict outcome, Lee et al (2004a) report two subclasses of
7   HCC patients characterized by significant differences in the length of survival and also identified
8   expression profiles of a limited number of genes that accurately predicted the length of survival.
9   Total RNAs from the 19 normal livers, including "normal liver in HCC patients," were pooled
10   and used as a reference for all microarray experiments and thus variations between patients and
11   especially differences due to conditions predisposing HCC, were not determined. DNA
12   microarray data using hierarchical clustering was reported to yield two major clusters, one
13   representing HCC tumors, and the other representing non-tumor tissues with a few exceptions
14   that were not characterized by the authors. Lee et al (2004a) report that along with 2 distinctive
15   subtypes of gene expression patterns in HCC there was heterogeneity among HCC gene
16   expression profiles and that one group had an overall survival time of 30.8 months and the other
17   83.7 months. Only about half the patients in each group were reported to have cirrhosis.
18   Expression of typical cell proliferation markers such as PCNA and cell cycle regulators such as
19   *CDK4, CCNB1, CCNA2 and CKS2* was greater in one class than the other of HCC.
20
21   The report by Boyault et al. (2007) attempted to compare etiology and genetic
22   characterization of the tumors they produce and confirms the heterogeneity of HCC, some
23   without attendant genomic instability.   Boyault et al. (2007) reported that genetic alterations are
24   indeed closely associated with clinical characteristics of HCC that define 2 mechanisms of
25   hepatocarcinogenesis. "The first type of HCC was associated with not only a high level of
26   chromosome instability and frequent TP53 and AXIN1 mutations but also was closely linked to
27   HBV infections and a poor prognosis. Conversely, the second subgroup of HCC tumors was
28   chromosome-stable, having a high incidence of activating β-catenin alteration and was not
29   associated with viral infection." Boyault et al (2007) reported that in a series of 123 tumors,
30   mutations in the CTNNB1 (encoding β-catenin), TP53, ACIN1, TCF1, PIK3CA and KRAS
31   genes in 34, 31, 13, 5, 2 and 1 tumors were identified, respectively. No mutations were found in
32   NRAS, HRAS, and EGFR. Hypermethylation of the CDKN2A and CDH1 promoter was
33   identified in 35% and 16% of the tumors, respectively. Boyault et al (2007) grouped tumors by
34   genomic expression as well as other factors. HCC groups associated with high rate of
35   chromosomal instability were reported to be enriched with over expression of cell-cycle/
36   proliferation/DNA metabolism genes. They concluded that "the primary clinical determinant of
37   class membership is HBV infection and the other main determinants are genetic and epigenetic
38   alterations, including chromosome instability, CTNNB1 and TP53 mutations, and parental
39   imprinting. Tumors related to HCV and alcohol abuse were interspersed across subgroups G3-
40   G6. Boyault et al (2007) suggested that there results indicate that HBV infection early in life
41   leads to a specific type of HCC that has immature features with abnormal parental gene
42   imprinting selections, possibly through the persistence of fetal hepatocytes or alternatively
43   through partial dedifferentiation of adult hepatocytes. "These G1 tumors are related to high-risk

1 populations found in epidemiological studies."

3.2. Animal Models of Liver Cancer

5 There are obvious differences between rodents and primate and human liver, and there is
6 a difference in background rates of susceptibility to hepatocarcinogenesis. With strains of mice
7 there are large differences in responses to hepatotoxins (e.g., acetaminophen) and to
8 hepatocarcinogens as well as background rates of hepatocarcinogenecity. Maronpot (2007)
9 reports that modulators of murine hepatocarcinogenesis, such as diet, hormones, oncogenes,
10 methylation, imprinting, and cell proliferation/apoptosis are among multiple mechanistically
11 associated factors that impact this target organ response in control as well as in treated mice, and
12 suggests that there is no one simple paradigm to explain the differential strain sensitivity to
13 hepatocarcinogenesis. Because of the variety of studies with differing protocols used to generate
14 susceptibility data, direct comparisons among strains and stocks is problematic but in regard to
15 susceptibility to carcinogenicity the C3H/HeJ and C57BL/6J mouse have been reported to have
16 up to a 40-fold difference in liver tumor multiplicity (Maronpot, 2007). However, as noted
17 above, TCE causes liver tumors in C6C3F1 and Swiss mice with studies of trichloroethylene
18 metabolites dichloroacetic acid, trichloroacetic acid, and chloral hydrate suggesting that both
19 dichloroacetic acid and trichloroacetic acid are involved in trichloroethylene-induced liver
20 tumorigenesis. Many effects reported in mice after dichloroacetic acid exposure are consistent
21 with conditions that increase the risk of liver cancer in humans and can involve GST Xi, histone
22 methylation, and overexpression of IGF2 (Caldwell and Keshava, 2006). The heterogeneity of
23 liver phenotype observed in mouse models is also consistent with human HCC. These data lend
24 support to the qualitative relevance of the mouse model for TCE-induced cancer risk.

26 Bannasch et al. (2003) made important observations that have implications regarding the
27 differences in susceptibility between rodent and human liver cancer. They stated that "Although
28 the classification of such nodular liver lesions in rodents as hyperplastic or neoplastic has
29 remained controversial, persistent nodules of this type are considered neoplasms, designated as
30 adenomas. In human pathology, the situation appears to be paradoxical because adenomas are
31 only diagnosed in the noncirrhotic liver, yet a confusing variety terms avoiding the clearcut
32 classification as an adenoma has been created for nodular lesions in liver cirrhoses, not
33 withstanding that the vast majority hepatocellular carcinomas develop in cirrhotic livers.   Even
34 if a portion of these nodular lesions would be regarded as adenomas, being integrated into an
35 adenoma-carcinoma sequence as observed in many animal experiments, clinical and
36 epidemiological records of liver neoplasms, including both benign and malignant forms, would
37 increase considerably. This would not only bring hepatic neoplasia further into focus of human
38 neoplasia in general, but also shed new light on the classification of some chemicals producing
39 high incidence of liver neoplasms in rodents, but appearing harmless to humans according to
40 epidemiological evaluations solely based on the incidence of hepatocellular carcinoma in
41 exposed populations." Thus, that in humans only HCCs are recorded but in animals adenomas
42 are counted as neoplasms, may indicate that the scope of the problem of liver cancer in humans
43 may be underestimated.

02/12/09

1   Tumor phenotype differences have been reported for several decades through the work of
2   Bannasch et al. The predominant cell line of foci of altered hepatocytes (FAH) have excess
3   glycogen storage early in development that appears to be similar to that shown by DCA
4   treatment. Bannasch et al. (2003) report that "the predominant glycogenotic-basophilic cell line
5   FAH reveals that there is an overexpression of the insulin receptor, the IGF-1 receptor, the
6   insulin receptor substrates-1/2 and other components of the insulin-stimulated signal transduction
7   pathway." Bannasch states that foci of this type have increased expression of GSTpi and insulin
8   has also been shown to induce the expression of GSTpi but that hyperinsulin-induced foci do not
9   show increased GSTpi. Cellular dedifferentiation during progression from glycogenotic to
10   basophilic cell populations is associated with downregulation in insulin signaling. The
11   amphophilic-basophilic cell lineage of peroxisome proliferators and hepadnaviridae were
12   reported to have foci that mimic effects of thyroid hormone with mitochondrial proliferation and
13   activation of mitochondrial enzymes. Bannasch et al. (2003) state that "the unequivocal
14   separation of 2 types of compounds, usually classified as initiators and promoters, remains a
15   problem at the level of the foci because at least the majority of chemical hepatocarcinogens seem
16   to have both initiating and promoting activity, which may differ in quantitative rather than
17   qualitative terms from one compound to another." "Whereas genetic mutations have been
18   predominantly postulated to initiate hepatocarcinogenesis for many years, more recently
19   epigenetic changes have been increasingly discussed as a plausible cause of the evolution of
20   preneoplastic foci characterized by metabolic changes including the expression of GSTpi."

21   Su and Bannasch (2003) report that glycogen-storing foci represents early lesion with the
22   potential to progress to more advance glycogen-poor basophilic lesions through mixed cell foci
23   and resulting hyperproliferative lesions and are associated with HCC in man. Small-cell change
24   (SCC) of liver parenchyma (originally called liver cell dysplasia of small cell size) is reported to
25   share cytological and histological similarities to early well defined HCC. Close association
26   between SCC and more advanced (basophilic) foci indicates that foci often progress to HCC
27   through SCC in humans. SCC were reported to be present in all basophilic foci.   Previous
28   studies were cited that showed that the biochemical phenotype of human FAH, mainly including
29   glycogen storing clear cell foci and clear cell-predominated mixed cell foci, were observed in
30   more than 50% of cirrhotic livers with or without HCC. FAH of clear and mixed cell types were
31   observed in almost all livers bearing HCC, and in chronic liver diseases without HCC but at a
32   lower frequency. Su and Bannasch (2003) report that "the finding of mixed cell foci (MCF)
33   mainly in livers with high-risk or cryptogenic cirrhosis indicates that these are more advanced
34   precursor lesions in man, in line with earlier observations in experimental animals. Considering
35   their preferential emergence in cirrhotic livers of the high-risk group, their unequivocally
36   elevated proliferative activity, and the resulting large size with frequent nodular transformation,
37   we suggest that mixed cell populations are endowed with a high potential to progress to HCC in
38   humans, as previously shown in rats." In human HCC, irregular areas of liver parenchyma with
39   marked cytoplasmic amphophilia, phenotypically similar to the amphophilic preneoplastic foci in
40   rodent liver exposed to different hepatocarcinogenic chemicals (e.g. DHEA a peroxisome
41   proliferator) or the hepadnaviruses were reported to present in 45% of the specimens from
42   cirrhotic livers examined. "However, more data are needed to elucidate the nature of the

oncocytic and amphophilic lesions regarding their role in HCC development."

With respect to the ability respond to a mitogenic stimulus, differences between primate and rodent liver response to a powerful stimulus, such as partial hepatectomy, have been noted that indicate that primate and human liver respond differently (and much more slowly) to such a stimulus. Gaglio et al. (2002) report after 60% partial hepatectomy in Rhesus macaques (Macaca mulatto), the surface area of the liver remnant was restored to its original preoperative value over a 30 day period. The maximal liver regeneration occurred between days 14 and 21, with thickening of liver cell plates, binucleation of hepatocytes, Ki-67 and PCNA expression (occurring in hepatocytes throughout the lobule at a maximum labeling index of 30%), and mitoses parallel increased most prominently between post-hepatectomy days 14 and 30. However, cytokines associated with inducing proliferation were elevated much earlier. TGF-α, IL-6, HGF, IL-6 and TNF-α mRNA persisted until day 14, with peak elevations of IL-6, TNF-α, occurring 24 hours later surgery, and IL-6 reduced to control levels by day 14. Gaglio et al. (2002) suggest that their results clearly indicate that the pattern and timing of liver regeneration observed in this non-human primate model are significantly different when comparing different species (e.g., peak expression of Ki-67 in a 60% partial hepatectomy model in rats occurs within hours following partial hepatectomy) and that the difference in timing and pattern of maximal hepatocellular regeneration cannot be explained simply by differences in size of animals (e.g., 60% partial hepatectomy in dogs produced liver regeneration peaks at 72 hours with weights approximating the weights of the Rhesus macaques). They note that previous studies in humans, who underwent 40-80% partial hepatectomy, reveal a similar delay in peak liver regeneration based on changes in serum levels of ornithine decarboxylase and thymidine kinase, further highlighting significant interspecies differences in liver regeneration. For C57BL/6 X 129 mice Fujita et al (2001) report that after partial hepatectomy, the liver had recovered more than 90% of its weight within 1 week. This difference in response to a mitogenic stimulus has impacts on the interpretations of comparisons between rodent and primate liver responses to chemical exposures which give a transient increases in DNA synthesis or cell proliferation such as PPARα agonists. Also, as stated above, the primate and human liver, while having a significant polyploidy compartment, do not have the extent of polyploidization and the early onset of that has been observed in the rodent. However, as noted by Lapis et al (1995), exposure to DEN has proven to be a highly potent hepatocarcinogen in non-human primates, inducing malignant tumors in 100% of animals with an average latent period of 16 months when administered at 40 mg/kg intraperitoneally every 2 weeks.

In regard to species extrapolation of epigenomic changes between humans and rodents, Weidman et al. (2007) caution that "Although we do predict some overlap between mouse and human candidate imprinted genes identified through our machine-learning approach, it is likely that the most significant criterion in species-specific identification will differ. This difference underscored the importance for increased caution when assessing human risk from environmental agents that alter the epigenome using rodent models; the molecular pathways targeted may be independent."

1   Despite species differences, the genome of the mouse has been sequenced and many
2   transgenic mouse models are being used to study the consequences of gene expression
3   modulation and pathway perturbation to study human diseases and treatments. However the use
4   of transgenic models must be used with caution in trying to determine to determine MOAs and
5   the background effects of the transgene (including background levels of toxicity) and specificity
6   of effects must be taken into account for interpretation of MOA data, especially in cases where
7   the knockout in the mouse causes significant liver necrosis or steatosis (Keshava and Caldwell,
8   2006; Keshava et al 2006; Caldwell and Keshava, 2006; Caldwell et al 2007b). For the
9   determination of effects of pathway perturbation and similarity to human HCC phenotype,
10   mouse transgenic models have been particularly useful with tumors produced in such models
11   shown to correlate with tumor aggressiveness and survival to human counterparts.
12
13   **3.2.1. Similarities with Human and Animal Transgenic Models**

14   Mice transgenic for transforming growth factor $-\alpha$ (a member of the EGF family and a
15   ligand for the ErfB receptors) develop HCCs (Farazi and DePinho, 2006). Compound TGFα and
16   MYC transgenic mice show increase hepatocarcinogenesis that is associated with the disruption
17   of TGFβ1 signaling and chromosomal losses, some of which are syntenic to those in human
18   HCCs that include the retinoblastoma (RB) tumour suppressor locus (Sargent et al 1999). Lee et
19   al. (2004b) investigated whether comparison of global expression patterns of orthologous genes
20   in human and mouse HCCs would identify similar and dissimilar tumor phenotypes, and thus
21   allow the identification of the best-fit mouse models for human HCC. The molecular
22   classification of HCC on the basis of prognosis in Lee et al. (2004a) was further compared with
23   gene-expression profiles of HCCs from seven different mouse models (Lee et al., 2004b). Lee et
24   al (2004b) characterized the gene expression patters of 68 HCC from 7 different mouse models;
25   two chemically induced (Ciprofibrate and diethylnitrosamine), four transgenic (targeted
26   overexpression of *Myc, E2F1, Myc and E2F1*, and *Myc* and *Tgfa* in the liver).   HCCs from some
27   of these mice (MYC, E2F1 and MYC-E2F1 transgenics) showed similar gene-expression
28   patterns to the ones of HCCs from patients with better survival. Murine HCCs derived for
29   MYC-TGFα transgenic model or diethylnitrosamine-treated mice showed similar gene-
30   expression patterns to HCCs from patients with poor survival. The authors report that Myc Tgfa
31   transgenic mice typically have a poor prognosis, including earlier and higher incident rates of
32   HCC development, higher mortality, higher genomic instability and higher expression of poor
33   prognostic markers (e.g., AFP) and that Myc and Myc/E2f1 transgenic mice have relatively
34   higher frequency of mutation in β-catenin (*Catnb*) and nuclear accumulation of β-catenin that are
35   indicative of lower genomic instability and better prognosis in human HCC.

36   Lee et al (2004b) indentified three distinctive HCC clusters, indicating that gene
37   expression pattern of mouse HCC are clearly heterogeneous and reported that Ciprorfibrate-
38   induced HCCs and HCCs from Acox-/- mice were closely clustered and well separated from
39   other mouse models. However, are several issues regarding this study that give limitations to
40   some of its conclusions regarding the Acox -/- mouse and Ciprofibrate treatment. The Acox-/-
41   mouse is characterized by profound hepatonecrosis which confounds conclusions regarding gene

1 expression related to PPARα agonism made by the authors. There was very limited reporting of
2 the animal models (DEN and Clofibrate) protocols used. Only 3 tumors were examined for
3 Clofibrate treatment and it is unknown if the tumors were from the same animals. Similarly only
4 3 tumors were examined from DEN treatment which has been shown to produce heterogeneous
5 tumors and to produce necrosis in some paradigms of exposure. Myc/E2F1 and E2F1 mice were
6 split in both clusters that were compared with human HCCs. The authors used previously
7 published data from Meyer et al. (2003) for tumors from Acox1[-1-] null mice, DENA-treated mice
8 and Ciprofibrate-treated mice.

9   Meyer et al (2003) examined three tumors from 2 C57BL/6j mice fed Ciprofibrate for 19
10 months and three tumors from 2 C57BL/6j mice injected with DEN at 2-3 months but the age at
11 which tumors appear was not given by the authors. Pooled mRNA from animals of varying age
12 (5 – 15 months old was used for controls. mRNAs that differed by 2-fold in tumors were
13 reported to be: 60 genes up-regulated and 105 genes down-regulated in Acox1[-1-] null mice
14 tumors; 136 genes up-regulated and 156 genes down-regulated in Ciprofibrate-induced tumors;
15 and 61 genes up-regulated and 105 genes down-regulated in DEN-induced tumors. The authors
16 state that "Each tumor class revealed a somewhat different unique expression pattern." There
17 were "genes that were general liver tumor markers in all three types of tumors" with 38 genes
18 commonly deregulated in all three tumor types. On note, the cell cycle genes (CDK4,
19 CDC25Am CDC7 and MAPK3) cited by Lee et al (2004b) as being more highly expressed in
20 DEN-induced tumors were not reported to be changed in DEN tumors in Meyer et al (2003) or to
21 be altered in the Acox1[-1-] null mice or mice treated with Ciprofibrate. Finally, the distinction
22 between groups may be dominated by gene expression changes in a large number of genes that
23 are related to PPAR activation but not related to hepatocarcinogenesis.

24   Calvisi et al. (2004a) used transgenic mice to study pathway alterations and tumor
25 phenotype and to further examine the premise that genomic alterations (genetic and epigenetic)
26 characteristic of HCC can describe tumors into 2 broad categories, the first category
27 characterized by activation of the Wnt/Wingless pathway via disruption of β-catenin function
28 and chromosomal stability and the second by chromosomal instability. Increased coexpression
29 of c-myc with TGF-α or E2F-1 transgenic mice was reported to result in a dramatic synergistic
30 effect on liver tumor development when compared with respective monotransgenic lines,
31 including shorter latency period, and more aggressive phenotype whereas β-catenin activation is
32 relatively common in HCCs developed in c-myc and c-myc/TGF-β1 transgenic mice, rare in the
33 c-myc/TGF-α transgenic line which also has genomic instability. Calvisi et al. (2004a) also
34 report that β-catenin staining correlated with histopathologic type of liver tumors. Eosinophilic
35 tumors with abnormal nuclear staining of β-catenin were predominant in neoplastic lesions
36 characteristic of c-myc and c-myc/E2F1 lesions. Poorly differentiated HCCs with basophilic or
37 clear-cell phenotypes developed more frequently in c-myc/TGF-α and TGF-α mice and often
38 showed a reduction or loss of β-catenin immunoreactivity. β-catenin mutation was associated
39 with a more benign phenotype. Calvisi et al. (2004a) note that the relationship between β-
40 catenin activation, tumor grade, and clinical outcome in human HCC remains controversial.

1   "There are studies that show a significant correlation between β-catenin nuclear accumulation, a
2   high grade of HCC tumor differentiation, and a better prognosis, whereas others find that nuclear
3   accumulation of β-catenin may be associated with poor survival or that it does not affect clinical
4   outcome."

5   Calvisi et al. (2004b) report for E-cadherin a variety of morphologenetic events, including
6   cell migration, separation and formation of boundaries between cell layers and differentiation of
7   each cell layer into functionally distinct structures. Loss of expression of E-cadherin was
8   reported to result in dedifferentiation, invasiveness, lymph node or distant metastasis in a variety
9   of human neoplasms including HCC and that the role of E-cadherin might be more complex that
10 previously believed. In order to elucidate the role of E-cadherin in the sequential steps of liver
11 carcinogenesis, we have analyzed the expression patterns of E-cadherin in a collection of
12 preneoplastic and neoplastic liver lesions from c-Myc, E2F1, c-Myc/TGF-α and c-Myc/E2F1
13 transgenic mice. In particular, we have investigated the relevance of genetic, epigenetic and
14 transcriptional mechanisms on E-cadherin protein expression levels. Our data indicate that loss
15 of E-cadherin contributes to HCC progression in c-Myc transgenic mice by promoting cell
16 proliferation and angiogenesis, presumably through the upregulation of HIF-1α and VEGF
17 proteins. The c-Myc line, was most like wild type and lost E-cadherin in the tumors. c-
18 Myc/TGF-α dysplastic lesion were reported to show overexpression of E-cadherin mainly in
19 pericentral areas with E2F1 clear cell carcinoma showed intense staining of E-cadherin. Since
20 reduction or loss of E-cadherin expression is primarily determined by loss of heterozygosity at
21 the E-cadherin locus or by its promoter hypermethylation in human HCC Calvisi et al (2004b)
22 determined the status of the E-cadherin locus and promoter methylation in wild type livers and
23 tumors from transgenic mice by microsatellite analysis and methylation specific PCR,
24 respectively. "Wild-type livers and HCCs, regardless of their origins, showed the absence of
25 LOH at the E-cadherin locus. E-cadherin promoter was not hypermethylated in wild-type, c-
26 Myc/TGF-α and E2F1 livers. No E-cadherin promoter hypermethylation was detected in c-Myc
27 and c-Myc/E2F1 HCCs with normal levels of E-cadherin protein. In striking contrast, seven of
28 20 (35%) of c-Myc and two of four (50%) c-Myc/E2F1 HCCs with downregulation of E-
29 cadherin displayed E-cadherin promoter hypermethylation. These results suggest that promoter
30 hypermethylation might be responsible for E-cadherin downregulation in a subset of c-Myc and
31 c-Myc/E2F1 HCCs." "The molecular mechanisms underlying down-regulation of E-cadherin in
32 c-Myc tumors remain poorly understood at present. No LOH at the E-cadherin locus was
33 detected in the c-Myc HCCs whereas only a subset of c-Myc tumors displayed hypermethylation
34 of the E-cadherin promoter. Furthermore, no association was detected between E-cadherin
35 downregulation and protein levels of transcriptional repressors, Snail, Slug or the tumor
36 suppressor WT1, in disagreement with the finding that overexpression of Snail suppresses E-
37 cadherin in human HCC." "E-cadherin might play different and apparently opposite roles, which
38 depend on specific tumor requirements in both human and murine liver carcinogenesis."
39 Importantly, the results of Calvisi et al. (2004b) that hypermethylation of promoters can be
40 associated with down regulation of a gene in mouse liver tumors similar to human HCC and that
41 tumors can have the same behaviour with methylation change as with loss of hetererozygosity.

1   This report also gives evidence of the usefulness of the mouse model to study human
2   liver cancer as it shows the similarity of dysfunctional regulation in mouse and human cancer
3   and the heterogeneity within and between mouse lines tumors with differing dysfunctions in
4   gene expression. This parallels human cancer where there is heterogeneity in tumors from one
5   person and every tumor has its own signature. Finally, this report correlates differing pathway
6   perturbations with mouse liver phenotypes similar to those reported in experimental
7   carcinogenesis models and for TCE and its metabolites.

8   Farazi and DePinho (2006) suggest that "as comparative array CGH analysis of various
9   murine cancers has shown that such aberrations often target syntenic loci in the analogous human
10   cancer type, we further suggest that comparative genomic analysis of available mouse model of
11   mouse HCC might be particularly helpful in filtering through the complex human cancer
12   genome. Ultimately mouse models that share features with human HCCs could serve as valuable
13   tools for gene identification and drug development. However, one needs to keep in mind key
14   differences between mice and humans. For example, as noted in certain human HCC cases,
15   telomere shortening might drive the genomic instability that enables the accumulation of cancer-
16   relevant changes for hepatocarcinogenesis. As mice have long telomeres, this aspect of
17   hepatocarcinogenesis might be fundamentally different between the species and provide additional
18   opportunities for model refinement and testing of this mechanism through use of a telomere
19   deficient mouse model. These and other cross-species difference, and limitations in the use of
20   human cell-culture systems, must be considered in any interpretation of data from various model
21   systems" (Farazi and DePinho, 2006). Thus, these mouse models of liver cancer inductions are
22   qualitatively able to mimic human liver cancer and support the usefulness of mouse models of
23   cancer.

3.3. Hypothesized Key Events in HCC Using Animal Models

3.3.1. Changes in ploidy

26   As stated above in Section 1.1, increased polyploidization has been associated with
27   numerous types of liver injury and appears to result from exposure to TCE and its metabolites as
28   well as changes in the number of binucleate cells. Hortelano et al (1995) reported that cytokines
29   and NO can affect ploidy and further suggests a role of these changes for carcinogenesis in
30   general. Vickers et al. (1996) noted that while both DEN and 17 α-ethinylestradiol have been
31   reported to enhance the proportion of diploid hepatocytes, initiators like N-nitrosomorpholine are
32   reported to increase the proportion of hypertrophied and polyploidy hepatocytes. The
33   relationship of such changes to cancer induction has been studied in transgenic mouse models
34   and in models involved with mitogens of differing natures.

35   Melchiorri et al. (1993) report the response pattern of the liver to acute treatment with
36   primary mitogens in regard to ploidy changes occurring in rat liver following two different types
37   of cell proliferation: compensatory regeneration induced by surgical partial hepatectomy and
38   direct hyperplasia induced by the mitogens lead nitrate and Nafenopin (a PPARα agonist) in 8

1 week old male Wistar rats. Feulgen stain was used and DNA content quantified by image
2 cytometry in mononucleate and binucleate cells. Mitotic index was determined in the same
3 samples. The term "diploid" was used to identify cells with a single, diploid nucleus and
4 tetraploid for cells containing 2 diploid nuclei or one tetraploid nucleus referred (bi- and
5 mononucleate, respectively). Octoploid cells were identified as either binucleate or
6 mononucleate. "During liver regeneration following surgical PH an increase in the mitotic index
7 with a peak at 24 hours was observed. The most striking effect associated with the regenerative
8 response was the almost complete disappearance of binucleate cells, tetraploid (2 X 2c) as well
9 as octoploid (4 X 2c) with only < 10% of the control values being present 3 days after PH."
10 "Concomitantly, an increase in mononucleate tetraploid (4c) as well as mononucleate octoploid
11 (8c) cells was observed, resulting at 3 days after PH in a population made up of almost entirely
12 (98%) by mononucleated cells." However, lead nitrate treatment was reported to induce rapid
13 increase in the formation of binucleate cells occurred with, 3 days after treatment, their number
14 accounting for 40% of the total cell population versus 22% binucleate cells in control rats and
15 2% in PH animals killed at the same time point. The increased binuclearity was reported to be
16 observed only in the 4 X 2c cells (25% vs. 6% of the controls) and in 8 X 2c cells (3.7% versus
17 0.1% of controls). The increase in 4 X 2c and 8 X 2c cells was reported to be accompanied by a
18 concomitant reduction in 2 X 2c cells with the change induced in cellular ploidy by lead nitrate
19 resulting in 37% of cells being either 8c or 16c. However, at the same time point, cells having a
20 ploidy higher than 4c were reported to account for only 11% in PH rats and 9% in control
21 animals. Changes in the ploidy pattern were reported to be preceded by an increased mitotic
22 activity which was maximal 48 hours after treatment with lead nitrate. The increase in mitotic
23 index in lead nitrate-treated rats was associated with a striking increase in the labeling index of
24 hepatocytes (60.1% versus 3% of control rats) and to an almost doubling of hepatic DNA content
25 in 3 days after lead nitrate. Melchiorri et al. (1993) concluded that the entire cell cycle appeared
26 to be induced by lead nitrate but that the finding of a high increase of binucleate cells suggested
27 that lead nitrate-induced liver growth, unlike liver regeneration induced by partial hepatectomy
28 was characterized by an uncoupling between cell cycle and cytokinesis which raised questions
29 whether lead nitrate-induced liver growth resulted in a true increase in cell number or is only the
30 expression of an increased hepatocyte ploidy. They reported that part of the increase in DNA
31 content observed 3 days after lead nitrate was indeed expression of polyploidizing process due to
32 acytokinetic mitoses but that a consistent increase in cells number (+26%) was also induced by
33 lead nitrate treatment.

34   After Nafenopin treatment, Melchiorri et al. (1993) reported that the increase in DNA
35 content was increased 22% over controls and was much lower than induced by lead nitrate and
36 that Nafenopin did not induce significant changes in binucleate cell number. However, a shift
37 towards a higher ploidy class (8c) was reported to be observed following Nafenopin and the 21%
38 increase in DNA content seen after Nafenopin treatment was almost entirely due to increase in
39 the ploidy state with only 7% increase in cell number. Melchiorri et al (1993) examined whether
40 hepatocytes characterized by high ploidy content (highly differentiated cells) would be
41 preferentially eliminated by apoptosis. An increase in apoptotic bodies was reported to be
42 associated with the regression phase after lead nitrate treatment (when liver mass is reduced) but

despite the elimination of excess DNA, the changes in ploidy distribution induced by lead nitrate were found to persist suggested that polyploidy cells were not preferentially eliminated by apoptosis during the regression phase of the liver. Melchiorri et al (1993) note that other studies in rat exposed to the mitogens cyproterone acetate (CPA) and the peroxisome proliferator methylclofenapate (MCP) also reported a very strong decline in binucleate cells with a concomitant increase in mononucleate tetraploid cells in the liver similar to the pattern described after partial hepatectomy.

Lalwani et al. (1997) reported the results of 1000 ppm WY-14,643 exposure in male Wistar rats after 1, 2, and 4 weeks and suggested that an early wave of nuclear division occurred at the early stages of exposure without cumulative effects on cell proliferation. Consistent with hepatomegaly, WY-14,643-treated were reported to exhibit multifocal hepatocellular hypertrophy and karyomegaly by routine microscopic analysis. For binucleate hepatocytes, there were no reported differences between WY-14,643 and controls for days 4 and 11 but an increase in the number at day 25 in WY-14,643-treated animals compared to controls. Increases in the diameter of nuclei were shown by WY-14,643 treatment from Day 11 and 25 with increasing numbers of cells displaying larger nuclear diameters. The mitotic index was reported to not be significantly changed in WY-14,643 treated rats compared to controls. Mitotic figures did not appear to survive the treatment necessary for flow cytometric analyses. Proliferating nuclear cell antigen (PCNA) was increased on day 4 in WY-14,643- treated animals compared to controls whereas no differences were found on days 11 and 25. However, immunohistochemistry was reported to show remarkable increases in BrdU-labeled nuclei in liver sections after 4 days of labeling with the populations of BrdU-labeled cell declining over the course of treatment. The labeling index was high and approximately 80% of the BrdU-labeled cells were in periportal areas. PCNA-expressing cells were increased in the periportal area of the liver. Intense nuclear staining of PCNA was evident as an indicator of DNA replication in S phase. Microscopic examination showed BrdU labeling only in periportal hepatocytes, whereas no significant labeling was observed in non-parenchymal cells, indicating that the replicative activity was confined to the liver cells. Lalwani et al (1997) suggested that their results showed that events related to cell proliferation occur in the initial phase of WY-14,643 treatment in rats but not followed by changes in the rate of DNA synthesis as the treatment progressed. They note that Marsman et al (1988) observed constant increases in DNA synthesis by [^{3}H]-thymidine authoradiography with up to 1 year of continuous administration of WY-14,643, whereas the rate of DNA synthesis or the BrdU labeling index in their study declined after the first 4 weeks of treatment. They suggest that the increased percentage of cells appearing in G2-M phase and the analysis of liver nuclear profiles suggest that the progression of these additional cells (i.e., cells that are stimulated to enter the cell cycle by the test agent) through the cell cycle is arrested in the late stages of the cell cycle. "Unlike BrdU labeling, which demonstrated DNA synthesis activity over the 4-day labeling period, the PCNA labeling index represents levels of the protein product at an interval post treatment. PCNA expression in cells exposed to chemicals or to WY may not provide true representation of S phase or proliferative activity because PCNA-expressing nuclei were also found in G0=G1 and G2-M phases." Lalwani et al. (1997)

1   concluded that cell proliferation alone does not appear to constitute a determining process
2   leading to tumors in most tissues and sustained cell replication may not be a primary feature of
3   peroxisome proliferator-induced hepatocarcinogenesis. Miller et al. (1996) note that studies
4   with methylclofenapate (MCP) in Alpk:AP rats indicate that DNA synthesis occurs primarily in
5   one hepatocyte subpopulation as defined by ploidy status, the binucleated tetraploid (2 X 2N)
6   hepatocytes and that this preferential hepatocyte DNA synthesis is manifested by dramatic
7   alterations in hepatocyte ploidy subclasses, i.e., significant increases in mononucleate tetraploid
8   (4N) hepatocytes concomitant with decreases in 2 X 2N hepatocytes. They reported results in
9   male Fischer 344 rats were 13 weeks old (an agent in which polyploidization had reached a
10   plateau) exposed to 1000 ppm WY-14,643 and MCP (gavage via corn oil at 8 mg/ml or 25
11   mg/kg MCP once daily) for 2, 5, and 10 days (n = 4). WY-14,643 and MCP were reported to
12   induce significant increases in the octoploid hepatocyte class that coincided with decreases in the
13   tetraploid hepatocyte class. However, MCP did not induce this shift until day 5 of exposure.
14   These results show an approximate doubling of 8N hepatoctyes but still a very low number of the
15   total hepatocyte population that does not reach greater than 7% and is still only ~ twice that of
16   control values and thus does not present itself with a very large target population. There was no
17   real effect on 4N hepatocytes due to these treatments and the % of hepatocytes that were 4 N
18   stayed ~ 70% and were thus the majority cell type in the liver. Miller et al (1996) note the
19   importance of maturation and/or strain for these analyses there are maturation-dependent
20   differences in the distribution and mitogenic sensitivity of hepatoctyes in the various subclasses.
21   Hasmall and Roberts (2000) note that despite their differing hepatocarcinogenecity, both
22   DCB (a nonhepatocarcinogen in Fischer 344 rats) and DEHP at the doses and routes used in the
23   NTP bioassays induced similar profiles of S-phase labeling index (LI). A large and rapid peak
24   during the first 7 days (1115% and 1151% of control for DEHP and DCB, respectively) was
25   followed by a return to control levels. They suggest that the size of the S-phase response does
26   not necessarily determine hepatocarcinogenic risk and that the subpopulation in which S-phase is
27   induced may be a better correlate with subsequent hepatocarcinogenecity. They compared the
28   effects on polyploidy/nuclearity and on the distribution of S-phase labeled cells with ETU, the
29   peroxisome proliferator MCP, and phenobarbitone. Male F334 rats 7-9 weeks old were exposed
30   to MCP (0.1% in diet), ETU 83 ppm diet, phenobarbitone (500 mg/ml drinking water) for 7 days.
31   The number of rats for 7 day study was not given by the authors. Hasmall and Roberts (2000)
32   reported that treatment of rats with MCP, ETU or phenobarbitone for 7 days had no significant
33   effect on the ploidy profile as compared with corn oil controls (data not shown) but that MCP
34   and phenobarbitone did induce significant changes in nuclearity. MCP reduced the binuclear
35   tetraploid (2 X 2N) population and increased the mononuclear octoploid (8N) population.
36   Phenobarbitone similarly increased the proportion of cells in the 4N population. ETU had no
37   effect on the nuclearity profile as compared with control. However, what the authors describe for
38   their results in polidy and nuclearity are different than those presented in their figures. There
39   were significant differences between controls that the authors did not characterize and there
40   appeared to be a greater difference between controls than some of the treatments.
41
42   Gupta et al. (2000) report that in transgenic mice with overexpression of transforming
43   growth factor α (TGF α), liver-cell turnover increases, along with the onset of hepatic

1 polyploidy, whereas hepatocellular carcinoma originating in these animals contain more diploid
2 cells and notes that Co-expression of c-Myc and TGF α transgenes in mouse hepatocytes was
3 associated with greater degrees of polyploidy as well as increased development of hepatocellular
4 carcinoma. Gupta notes that in the presence of ongoing liver injury and continuous depletion of
5 parenchymal cells, hepatic progenitor cells (including oval cells) are eventually activated but
6 what roles polyploid cells play in this process requires further study. In the working model by
7 Gupta, sustained disease by chronic hepatitis, metabolic disease, toxins, ect., may lead to
8 hepatocyte polyploidy and loss, and the emergence of rapidly cycling progenitor or escape cell
9 clones with the onset of liver cancer.

10 Conner et al. (2003) describe the development of transgenic mouse models in which
11 E2F1 and/or c-Myc was overexpressed in mouse liver. The E2F1 and c-Myc transcription
12 factors are both involved in regulating key cellular activities including growth and death and,
13 when overexpressed, are capable of driving quiescent cells into S-phase in the absence of other
14 mitogenic stimuli and are potent inducers of apoptosis operating at least through one common
15 pathway involving p53. Deregulation of their expression is also frequently found in cancer cells
16 (Conner et al., 2003). Conner et al. (2003) reported that although both c-Myc and E2F1 mono-
17 transgenic mice were prone to liver cancer, E2F1 mice developed HCC more rapidly and with a
18 higher frequency and that the combined expression of these two transcription factors
19 dramatically accelerated HCC growth compared to either E2F1 or c-Myc mono-transgenic mice.
20 All three transgenic lines were reported to show a low but persistent elevation of hepatocyte
21 proliferation before an onset of tumor growth. Ploidy was shown to be affected differently by c-
22 Myc and E2F1, and suggested distinct differences by which these two transcription factors
23 control liver proliferation/maturation. Both transgenic alterations induced liver cancer but had
24 differing effects on polyploidization suggestive that liver cancer can arise from either type of
25 mature hepatocyte.

26 c-Myc single-transgenic mouse showed a continuous high cell proliferation preceded the
27 appearance of pre-neoplastic lesions which was also true, although to a lesser extent, in the E2F1
28 mouse. At 15 weeks of age, all of the transgenic mouse lines were reported to have a high
29 incidence (>60%) of hepatic dysplasia with mitotic indices were equivalent in c-Myc/E2F1, and
30 c-Myc livers, but 2-fold higher than the mitotic index in E2F1 and very low in wild type mice.
31 Thus, the combination of the two transgenes did not have and additive effect on proliferation.
32 An analysis of the DNA content in hepatocyte nuclei isolated from 4- to 15- week old mice was
33 reported to show that in young wild type livers, the majority of nuclei had a diploid DNA content
34 with a smaller proportion of tetraploid nuclei and as the mice aged, the number of tetraploid and
35 octoploid nuclei increased consistent with the previous findings of others. However, c-Myc mice
36 were reported to demonstrate a premature polyploidization with the number of 2N nuclei in c-
37 Myc livers was almost 2-fold less, while the proportion of 4N nuclei increased more than 2.5
38 fold at 4 weeks of age with the most prominent ploidy alteration being an increase in the fraction
39 of hepatocytes with octaploid nuclei (~ 200-fold higher). The percentage of polyploidy cells was
40 reported to continue to rise in 15 week old c-Myc livers with the majority of hepatocytes having
41 nuclei with 4N and 8N DNA content, with an attendant increase in binucleated hepatocytes and

1   increase in average cell size. In striking contrast, E2F1 hepatocytes were reported to not undergo
2   normal polyploidization with aging. The majority of E2F1 nuclei were reported to remain in the
3   diploid state and to be almost identical in E2F1 mice at 4 and 15 weeks of age with the
4   percentage of binucleated hepatocytes also reduced. In c-Myc/E2F1 mice, the age-related
5   changes in ploidy distribution were reported to resemble those found in both c-Myc and in E2F1
6   single transgenic mice. At a young age, c-Myc/E2F1 mice, similar to E2F1 mice, were reported
7   to retain significantly more diploid nuclei than c-Myc mice. However, as mice aged, the majority
8   of c-Myc/E2F1 hepatocytes, similar to c-Myc cells but in contrast to findings in E2F1 cells,
9   became polyploid. Consistent with a more progressive polyploidization, the DNA content was
10 significantly higher in both c-Myc/E2F1 and c-Myc livers. Conner et al. (2003) report that other
11 known modulators of ploidy in the liver are the tumor suppressor p53, pRb, and the cell cycle
12 inhibitor p21 as well as, genes involved in the control of the cell cycle progression such as cyclin
13 A, cyclin B, cyclin D3, and cyclin E.

14 Along with increased liver cancer, Conner et al. (2003) note that the C-Myc mice also
15 experienced a persistent liver injury as evidenced by significant elevation of circulating levels of
16 aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase along with the
17 appearance of a frequent oval/ductular proliferation. However, oval cell proliferation may be a
18 marker of hepatocyte damage but not be the cells responsible for tumor induction (Tarsetti et al.,
19 1993). Conner et al. (2000) report that in E2F1 is overexpressed in the liver there is both
20 oncogenic and tumor-suppressive properties. In regard to liver morphological changes, E2F1
21 transgenic mice were reported to uniformly develop pericentral dysplasia and foci adjacent to
22 portal tracts followed by the abrupt appearance of adenomas and subsequent malignant
23 conversion with all of the animals having foci by 2-4 months and by 8-10 months most having
24 adenomas with dysplastic changes remaining confined to the pericentral regions of the liver
25 lobule. In regard to phenotype, the majority of the foci were composed of small round cells, with
26 clear-cell phenotype but eosinophilic, mixed and basophilic foci were also seen. In adenomas
27 with malignant transformation to HCC, there appeared to be high mitotic indices, blood vessel
28 invasion and central collection of deeply basophilic cells with large nuclei giving a "nodule- in-
29 nodule: appearance. Macrovesicular hepatic steatosis was first noted in some E2F1 transgenic
30 livers at 6-8 months and by 10-12 months 60% of animals had developed prominent fatty
31 change. Hepatic steatosis has been noted in several transgenic mouse models of liver
32 carcinogenesis (Conner et al., 2000).   These results raise interesting points of regional difference
33 in tumor formation which can be lost in analyses using whole liver and that the phenotype of foci
34 and tumors are similar to those seen from chemical carcinogenesis. The occurrence of
35 hepatotoxicity in these transgenic mice is also of note.

36 **3.3.2 Hepatocellular Proliferation and Increased DNA Synthesis**
37
38 Caldwell et al (2007b) have presented a discussion of the role of proliferation in cancer
39 induction. They state that "in the case of CCl4 exposure, hepatocyte proliferation may be related
40 to its ability to induce liver cancer at necrogenic exposure levels, but the nature of this
41 proliferation is fundamentally different from peroxisome proliferators or other primary mitogens

1 that cause hepatocyte proliferation without causing cell death (Coni et al., 1993; Ledda-
2 Columbano et al., 1993, 1998, 2003; Menegazzi et al., 1997; Columbano and Ledda-Columbano,
3 2003).   After initiation with a mutagenic agent, the transient proliferation induced by primary
4 mitogens has not been shown to lead to cancer-induction, while partial hepatectomy or
5 necrogenic treatments of CCl4 result in the development of tumors [Ledda-Columbano et al.,
6 1993; Gelderblom et al., 2001]."   Roskams et al (2003) notes that partial hepatectomy does not
7 cause hepatocellular carcinoma in normal mice without initiation. Melchiorri et al (1993) report
8 that a series of studies has shown tha acute proliferative stimuli provided by primary mitogens,
9 unlike those of the regenertative type such as those elicited by surgical or chemical partial
10 hepatectomy, do not support the initiation phase and do not effectively promote the growth of
11 initiated cells (Columbano et al 1990; Columbano et al 1987; Ledda-Columbano et al 1989).
12 They note that, the finding that most of these chemicals, with the exception of WY, induce only a
13 very transient increase in cell proliferation raises the question whether such a transient induction
14 of liver cell proliferation might be related to liver cancer appearing 1-2 years later. They note
15 that mitogen-induced liver growth differs from compensatory regeneration in several aspects (1)
16 it does not require an increased expression of hepatocyte growth factor mRNA in the liver (2) it
17 is not necessarily associated with an immediate early genes such as c-fos and c-jun; (3) it results
18 in an excess of tissue and hepatic DNA content that is rapidly eliminatated by apoptotic cell
19 death following withdrawls of the stimulus.

20   Other studies have questioned the importance of a brief wave of DNA synthesis in
21 induction of cancer. Chen et al (1995) note that Jirtle et al. (1991) and Schulte-Hermann et al.
22 (1986) reported that during a 2-week period of treatment with lead, DNA synthesis was
23 increased most in centrolobular hepatocytes and that the predominantly centrilobular distribution
24 of the labeled nuclei may have been due largely to the brief wave of mitogenic response, because
25 from the fifth day onward DNA synthesis activity returned to control level even though lead
26 nitrate treatment continued. They concluded that sustained cell proliferation may ben more
27 important than a brief wave of increased DNA synthesis. Chen et al. (1995) reported that a
28 number of different agents acting via differing MOAs will induce periportal proliferation.
29 Vickers et al. (1996) reported that mitogenic response induced by acute 17 α-ethinylestradiol
30 adminstration is randomly distributed throughout the hepatic lobule, while continuous
31 adminstration increases the proportion of diploid cells. Richardson et al. (1986) reported that the
32 lobular distribution in the correlation of hepatocyte inititiation and akylation reported in their
33 model of carcinogenicity did "not support that early proliferation is associated with cancer as at 7
34 days there is a transient increase in the lobes least likely to get a tumor and no difference
35 between the lobes at 14 and 28 days DEN although there is a difference in tumor formation
36 between the lobes." Cells undergoing DNA synthesis may not be in the same zone of the liver
37 where other hypothesized "key events" take place. Tanaka et al. (1992) note that the distribution
38 of hepatocyte proliferation in the periportal area was in contrast to the distribution of peroxisome
39 proliferation in the centrilobular area of Clofibrate treated rats. Melnick et al. (1996) note that
40 replicative DNA synthesis commonly has been evaluated by measurement of the fraction of cells
41 incorporating bromodeoxyuridine or tritiated thymidine into DNA during S-phase of the cell
42 cycle (S-phase labeling index) but that the S-phase labeling index would not be identical to the
43 cell division rate when replication of DNA does not progress to formation of two viable daughter

1 cells. "The general view at an international symposium on cell proliferations and chemical
2 carcinogenesis was that although cell replication is involved inextricably in the development of
3 cancers, chemically enhanced cell division does not reliably predict carcinogenicity (Melnick et
4 al ,1993)." They note tha the finding that enzyme-altered hepatic foci were not induced in rats
5 fed WY-14,643 for 3 weeks followed by partial hepatectomy indicates that early high levels of
6 replicative DNA synthesis and peroxisome proliferation are not sufficient activities for initiation
7 of hepatocarcinogenesis.Baker et al., (2004) reported that, similar to the pattern of transient
8 increases in DNA synthesis reported for TCE metabolites, Clofibrate exposure induced the
9 upregulation of a variety of cell prolifertation-associated genes (e.g., G2/M specific cyclin B1,
10 cyclin-dependent kinase 1, DNA topoisomerase II alpha, c-myc protooncogene, pololike serien-
11 threonine protein kinase, and cell divisions control protein 20) began on or before day 1 and
12 peaked at some point between days 3 and 7. By day 7, cell proliferation genes were down
13 regulated.   The chronology of this gene expression agrees with the histologic dianosis of mitotic
14 figures in the tissue, where an increase in mitotic figures was detected in the day 1 and most
15 notably day 3 high and low-dose groups. However, by day 7, the incidence of mitotic figures
16 had decreased. The clustering of genes associated with the G2/M transition point suggests that in
17 the rats, the polyploidy cells arrested at G2/M are those that are proceeding through the cell
18 cycle.

19   A dose-response for increased DNA-synthesis also seems to be lacking for the model
20 PPARα agonist, WY-14,643 suggesting that the transient increase reported by Eacho et al (1991)
21 for this compound that then increases later at necrogenic exposure levels, are not related to its
22 carcinogenic potential. Wada et al. (1992) reported that in male Fischer 344 rats exposed to a
23 range of WY-14,643 concentrations (5 – 1000 ppm) that liver weight gain at the lowest dose
24 gave a sustained response for many weeks and gave increased cell labeling in the first week.
25 Peroxisomes proliferation as measure by electron microscopy increases started at 50 ppm
26 exposures but by enzymatic means, peroxisomal activities were elevated at the 5 ppm dose. Of
27 note is the difference in distribution in hepatocellular proliferation which was not where the
28 hypertrophy or where the lipofuscin increases were observed. The authors note that these data
29 suggest that 50 and 1000 ppm WY-14,643 should give the same carcinogencity if peroxisome
30 proliferation or sustained proliferation are the "key events." The study of Marsman et al (1992)
31 is very important in that it not only shows that clofibric acid dose not have sustatined
32 proliferation, but it also shows that it and WY-14,643 at 50 ppm did not induced apoptosis in
33 rats. It is probably that use of WY-14,643 are high concentrations may induce apoptosis in a
34 manner not applicable to other peroxisome proliferators or to treatment with WY-14,643 at 50
35 ppm. This study also confirms that exposure to WY-14,643 at 50 ppm and WY-14,643 at 1000
36 ppm induces similar effects in regards to hepatocyte proliferation and peroxisomal proliferation.
37
38   The study by Eacho et al. (1991) also gives a reference point for the degree of
39 hepatocytes undergoing transient DNA synthesis from WY-14,643 and Clofibrate and how much
40 smaller it is for TCE and its metabolites which generally involve less than 1% of hepatocytes.
41 "The labeling index of BrdU was 7.2% on day 3 and 15.5% on dayt 6 after clofibric acid but by
42 day 10 and 30 labeling index was the same as controls at ~1-2%." "For WY the labeling index
43 was 34.1% at day 3 and 18.6% at day 6. At day 10 the labeling index was 3.3% and at day 30

1 was 6%, representing 6.6- and 15-fold of respective controls. Control levels were ~ 0.5 to 1%."
2 "The labeling index was increased to 32% by 0.3% LY171883 and to 52% by 0.05% Nafenopin.
3 The 0.005% and 0.1% dietary doses of WY increased the 7 day labeling index to a comparable
4 level (55% - 58%)."
5
6 Yeldani et al. (1989) report results showing that until foci appear, cell proliferation has
7 ceased to increase over controls after the first week for ciprofibrate-induced
8 hepatocarcinogenesis and also shows the importance of using age matached controls and not
9 pooled controls for comparative purposes of proliferation as well as how low proliferative rates
10 are in control animals. The results of Barass et al. (1993) are important in suggesting when age
11 of animals is important when doing quantitation of labeling indexes. Studies such as that
12 conducted by Pogribny et al. (2007) that only give the replication rate as a ratio to control will
13 make the proliferation levels look progressive when in fact they are more stable with time, it is
14 just the controls that change with age as a comparison point.
15

3.3.3. Nonparenchymal Cell Involvement in Disease States Including Cancer

17
18 The recognition that not only parenchymal cells but also nonparenchymal cells play a
19 role in HCC has resulted in studies of their role in initiation as well as progression of neoplasia.
20 The role of the endothelial cell in controlling angiogenesis, a prerequisite for neoplastic
21 progression, and the role of the Kupffer cell and its regulation of the cytokine milieu that
22 controls many hepatocyte functions and responses have been reported. However, as pointed out
23 by Pikarsky et al. (2004) and by the review by Nickoloff et al. (2005) the roles of inflammatory
24 cytokines in cancer are context and timing specific and not simple. For TCE, non-parenchymal
25 cell proliferation has been observed after inhalation (Kjellstrand et al., 1983b) and gavage (Goel
26 et al., 1992) exposures of ~ 4 weeks duration.
27

3.3.3.1. Epithelial cell control of liver size and cancer – angiogenesis

29
30 The epithelium is key in controlling restoration after partial hepatectomy and not
31 surprisingly HCC growth.   Greene et al (2003) hypothesized that the control of physiologic
32 organ mass was similar to the control of tumor mass in the liver and that specifically, the
33 proliferation of hepatocytes after partial hepatectomy, like the proliferations of neoplastic cells in
34 tumors, requires the synthesis of new blood vessels to support the rapidly increasing mass. They
35 report that a peak in hepatocyte production of VEGF, an endothelial mitogen, corresponds to an
36 increase of VEGF receptor expression on endothelial cells after partial hepatectomy and the rate
37 of endothelial proliferation.. Fibroblast growth factor and transforming gowth factor-alpha,
38 which stimulate endothelial cells, are secreted by hepatoctyes 24 hours after partial hepatectomy
39 but endothelial cells were reported to secrete hepatocyte growth factor, a potent heaptocyte
40 mitogen that is also proangiogenic. The secretion of transforming growth factor –beta by
41 endothelial cells 72 hours after partial hepatectomy was reported to inhibit hepatocyte
42 proliferation. Thus, Greene et al. (2003) suggested that endothelial cells and hepatocytes of the
43 regenerating liver influence each other, and both populations are required for the regulation of

1   the regenerative process.

2

3   *3.3.3.2. Kupffer cell control of proliferation and cell signals, role in early and late effects*

4

5   Vickers et al. (1996) have rported that Kupffer cells are increased in number in
6   prenoplastic foci but are decreased in hepatocellular carcinom and that other studies have
7   demonstrated that both sinusoidal endothelial cells and Kupffer cells within hepatocellular
8   carcinoma cells in humans stain positive for mitotic activity although the number of non-
9   parenchymal cells compared to parenchymal cells may be reduced. Lapis et al. (1995) reported
10 that Kupffer cells contain lysozyme in their cytoplasmic granules, vacuoles and phagosomes and
11 some cells show a positive reaction in the rough endoplasmic reticulum, perinuclear cisternae
12 and the Golgi zone and that in human monocytes the lysozyme is co-localized with the CD68
13 antigen and myeloperoxidase. They also report that in rodent hepatocarcinogenesis increased
14 numbers of Kupffer cells were observed in preneoplastic foci, whereas abnormally low numbers
15 were present following progression to hepatocellular carcinoma.and that "the Kupffer cell count
16 in human HCC has also been shown to be very low and varies with different histological form."
17 They reported that for monkey HCCs, that the proportion of endothelial elements remained
18 constant (the parenchymal/endothelial cell ratio), however there was a striking reduction in the
19 areas occupied by Kupffer cells.   While healthy control livers contained the highest number of
20 Kupffer cells, in the tumor-bearing cases the non-neoplastic, non-cirrhotic liver adjacent to the
21 HCC nodules had a significantly lower number of Kupffer cells and the number decreased
22 further in the non-neoplastic portions of cirrhotic livers. Within HCC nodules the Kupffer cell
23 count was greatly reduced with no significant changes were observed between the cirrhotic areas
24 and the carcinomas, however, the tumors contrained fewer lysozyme and CD68 positive cells.
25 Lapis et al. (1995) note that "since other cell types withing the liver sinusoids (monocytes and
26 polypmorphs) and portal macrophage were also positive, it was important to identify the star-like
27 morphology of the Kupffer cells. The results of the two indepent observers assessment of the
28 morphology and enumeration of Kupffer cells were quite consistent and differed by only 3%."
29 "The loss of Kupffer cells in the HCC may possibly result from capillarization of the sinusoids,
30 which has been observed during the process of liver cirrhosis and carcinogenesis. Capillarization
31 entails the sinusoidal lining endothelial cells losing their fenestrations."

32

33 *3.3.3.3. Nf-kB and TNF-α - context, timing and source of cell signaling molecules*

34

35   A large body of literature has been devoted to the study of nuclear factor κ B for its role
36 not only in inflammation and a large number of other processes, but also for its role in
37 carcinogenesis. However, the effects of these cytokines are very much dependent on their
38 cellular context and the timing of their modulation. As described by Adli and Baldwin (2006),
39 "The classic form of NF-kB is composed of a heterodimer of the p50 and p65 subunits, which is
40 preferentially localized in the cytoplasm as an inactive complex with inhibitor proteins of the IkB
41 family. Following exposure to a variety of stimuli, including inflammatory cytokines and LPS,
42 IkBs are phosphorylated by the IKKα/β complexes then accumulate in the nucleus, where they
43 transcriptionally regulate the expression of genes involved in immune and inflammatory

1   responses." The five members of the mammalian NF-kB family, p65 (RelA), RelB, c-Rel,
2   P50/p105 (NF-KB1) and p52/p100 (NF-kB2), exist in unstimulated cells as homo- or
3   heterodimers bound to IkB family proteins. Transcriptional specificity is partially regulated by
4   the ability of specific NF-kB dimmers to preferentially associate with certain members of the IkB
5   family. Individual NF-kB responses can be characterized as consisting of waves of activation
6   and inactivation of the various NF-kB members (Hayden and Ghosh, 2004). While the function
7   of NF-kB in many contexts have been established, it is also clear that there is great diversity in
8   the effects and consequences of NF-kB activation with NF-kB subunits not necessarily
9   regulating the same genes in an identical manner and in all of the different circumstances in
10   which they are induced. The context within which NF-kB is activated, be it the cell type or the
11   other stimuli to which the cell is exposed, is therefore a critical determinant of the NF-kB
12   behavior (Perkins and Gilmore, 2006).
13
14   Balkwill et al. (2005) report that "the NF-κB pathway has dual actions in tumor
15   promotion: first by preventing cell death of cells with malignant potential, and second by
16   stimulating production of proinflammatory cytokines in cells of infiltrating myeloid and
17   lymphoid cells. The proinflammatory cytokines signal to initiated and/or otherwise damaged
18   epithelial cells to promote neoplastic cell proliferation and enhance cell survival. However, the
19   tumor promoting role of NF-κB may not always predominate. In some cases, especially early
20   cancers, activation of this pathway may be tuor suppressive (Perkins, 2004). Inhibiting NF-κB in
21   keratinocytes promotes squamous cell carcinogenesis by reducing growth arrest and terminal
22   differentiation of initiated keratinocytes (Seitz et al., 1998)." Other inflammatory mediators have
23   also been associated with oncogenesis. Balkwill et al. (2005) reported that TNFα is frequently
24   detected in human cancers (produced by epithelial tumor cells, as in for instance, ovarian and
25   renal cancer) or stromal cells (as in breast cancer). They also report that the loss of hormonal
26   regulation of IL-6 is implicated in the pathogenesis of several chronic diseases, including B cell
27   malignancies, renal cell carcinoma, and prostate, breast, lung, colon, and ovarian cancers. Over
28   100 agents, such as antioxidants, proteosome inhibitors, NSAIDs, and immunosuppressive
29   agents are NF-κB inhibitors with none being entirely specific (Balkwill et al., 2005). Thus,
30   alterations in these cytokines, and the cells that produce them, are implicated as features of
31   "cancer" rather than specific to HCC.
32
33   Balkwill et al. (2004) report that "Two mouse models of inflammation-associated cancer
34   now implicate the gene transcription factor NF-κB and the inflammatory mediator known as
35   tumour-necrosis factor α (TNF- α) in cancer progression. Using a mouse model of inflammatory
36   hepatitis that predisposes mice to liver cancers, Pikarsky et al. present evidence that the survival
37   of hepatocytes - liver cells - and their progression to malignancy, are regulated by NF-κB. NF-
38   κB is an important transcription factor that controls cell survival by regulating programmed cell
39   death, proliferation and growth arrest. Pikarsky et al. find that the activation state of NF-κB, and
40   its localization in the cell, can be controlled by TNF-α produced by neighboring inflammatory
41   cells (collectively known as stromal cells)." Pikarsky et al (2004) reported that that the
42   inflammatory process triggers hepatocyte NF-κB through upregulation of TNF-α in adjacent
43   endothelial and inflammatory cells. Switching off NF-κB in mice from birth to seven months of

1 age, using hepatocyte-specific inducible IκB-super repressor transgene, had no effect on the
2 course of hepatitis, nor did it affect early phases of hepatocyte transformation. By contrast,
3 suppressing NF-κB inhibition through anti- TNF-α treatment or induction of the IκB-super
4 repressor in later stages of tumor development resulted in apoptosis of transformed hepatocytes
5 and failure to progress to hepatocellular carcinoma. The Mdr2 knockout hepatocytes in
6 Pikarsky's model of hepatocarcinogenicity were distinguishable from wild-type cells by several
7 abnormal features; high proliferation rate, accelerated hyperploidy and dysplasia. Pikarsky et al
8 (2004) reported that NF-κB knockout and double mutant mice displayed comparable degrees of
9 proliferation, hyperploidy and dysplasia implied that NF-κB is not required for early neoplastic
10 events. Thus, activation of NF-κB was not important in the early stages of tumour development,
11 but was crucial for malignant conversion. "Greten et al reporting in Cell, come to a similar
12 conclusion by studying a mouse colitis-asociated cancer model. Their work does not directly
13 implicate TNF-α, but instead found enhanced production of several pro-inflammatory mediators
14 (cytokines) including TNF-α,, in the tumour microenvironment during the development of
15 cancer. An important feature of both studies is that NF-κB activation was selectively ablated in
16 different cell compartments in developing tumour masses, and at different stages of cancer
17 development." However, Balkwill et al (2004) also note that TNF-α and NF-κB have many
18 different effects, depending on the context in which they are called into play and the cell type
19 and environment.
20
21   In contrast, inhibition of Nf-κB impaired HCC progression in a mouse model of
22 cholestatic hepatitis (Pikarsky et al., 2004). El-Serag and Rudolph (2007) note that "the
23 influence of inflammatory signaling on hepatocarcingenesis can be context dependent; deletion
24 of Nf-κB-dependent inflammatory responses enhanced HCC formation in carcinogen treated
25 mice (Sakurai et al 2006). Similarly, deletion of Nf-κB essential modulator/ I kappa β kinase
26 (NEMO/IKK), an activator of Nf-κB, induced steatohepatitis and HCC in mice (Luedde et al.,
27 2007). Maeda et al. (2005) reported that hepatocyte specific deletion of IKKβ (which prevents
28 NF-kB activation) increased DEN-induced hepatocarcingenesis and that a deletion of IKKβ in
29 both hepatocytes and hematopoietic-derived cells, however, had the opposite effect, decreasing
30 compensatory proliferation and carcinogenesis. They suggest that thes results, which differ
31 from previous suggestion that the tumor-promoting function of NF-kB is excreted in hepatocytes
32 (Pikarsky et al., 2004) and suggest that chemicals or viruses that interfere with NF-kB activation
33 in hepatocytes may promote HCC development.
34
35   Alterations in NF-kB levels have been suggested as a key event for the
36 hepatocarcinogenicity PPARα agonists. The event associated with PPAR effects has been the
37 extent of NF-kB activation as determined through DNA binding. As reported by Tharappel et al
38 (2001), NF-kB activity is assayed with eletrophoretic modbility shift assay (EMSAs) with
39 nuclear extracts prepared from frozen live tissue as a measure of DNA binding of NF-kB.
40 Increase transcription of downstream targets of NF-kB activity have also been measured. It has
41 been suggested that PPARα may act as a protective mechanism against liver toxicity. Ito et al
42 (2007) cite repression of NF-kB by PPARα to be the rationale for their hypothesis that PPARα-
43 null mice may be more vulnerable to tumorigenesis induced by exposure to environmental

1   carcinogens. However, as shown in Section 3.4.1.2., although DEHP was reported to also induce
2   glomerularnephritis more often in PPARα-null mice as suggested Kamijo et al (2007) due of the
3   absence of PPARα- dependent anti-inflammatory effect of antagonizing the oxidative stresss and
4   NF-κB pathway, there was no greater or lesser susceptibility to DEHP-induced liver
5   carcinogencicity in the PPARα null mice.

6

7   Because PPARα is known to exert anti-inflammatory effects by inducing expression of
8   IκBα, which antagonizes NFκB signaling, the expression of IκBα has been measured in some
9   studies (Kamijo et al (2007) as well as expression of TNR1 mRNA to evaluate the sensitivity to
10 the inflammatory response. Ito et al (2007) report that in wild type mice there did not appear to
11 be a difference between controls and DEHP treatment for p65 immunoblot results. DEHP
12 treatment was also reported to not induce p65 or p52 mRNA either or influence the expression
13 levels of TNFα, IkBα, IkBβ and IL-6 mRNA in wild type mice. Tharappel et al. (2001) treated
14 with WY-14,643, gemfibrozil or Dibutyl phthalate in rats and reported elevated NF-kB DNA
15 binding in rats with WY-14,642 to have sustained response but not others. WY increased DNA
16 binding activity of NF-kB at 6, 34 or 90 days. Gemfibrozil and DEHP increased NF-kB activity
17 to a lesser extent and not at all times in rats. For gemfibrozil there was only a 2-fold increase in
18 binding at 6 days with no increase at 34 days and increase only in low dose at 90 days. In rats
19 treated with Dibutyl phthalate, there no change at 6 days, at 34 days there was an increase at high
20 and low dose, at 90 days only low dose animals showed a change. In pooled tissue from WY-
21 14,643- treated animals, the complex that bound the radiolabled NF-kB fragment did contain
22 both p50 and p65. Both WY-14,643 and gemfibrozil were reported to produce tumors in rats
23 with Dibutyl pthalate untested in rats for carcinogenicity. Thus, early changes in NF-kB were
24 not supported as a key event and WY-14,643 to have a pattern that differed from the other
25 PPARα agonists examined.

26

27   In regard to the links between inflammation and cancer, Nickoloff et al. (2005) in their
28 review of the issue, caution that such a link is not simple. They note that "dissecting the
29 mediators of inflammation in cutaneous carcinogenic pathways has revealed key roles for
30 prostaglandins, cyclooxygenase-2, tumor necrosis factor-α, AP-1, NF-κB, signal transducer and
31 activator of transcription (STAT)3, and others Several clinical conditions associated with
32 inflammation appear to predispose patients to increased susceptibility for skin cancer including
33 discoid lupus erythematosus, dystrophic epidermolysis bullosa, and chronic wound sites.
34 Despite this vast collection of data and clinical observations, however, there are several
35 dermatological setting associated with inflammation that do not predispose to conversion to
36 lesions into malaignancies such as psoriasis, atopic dermatitis, and Darier's disease." Nickoloff
37 et al. (2005) suggest that such a "link may not be as simple as currently portrayed because certain
38 types of inflammatory processes in skin (and possibly other tissues as well) may also serve a
39 tumor suppressor function. Over the past few months, several publications in leading biomedical
40 journals grappled with an important issue in oncology, namely defining potential links between
41 chronic tissue damage, inflammation, and the development of cancer. Balkwill and Coussens
42 (2004) reviewed the role of the NF-κB signal transduction pathway that can regulate
43 inflammation and also promote malignancy. Their review summarixed the latest findings

1 revealed in a letter to Nature by Pikarsky et al. (2004). Using Mdr2 knockout mice in which
2 hepatitis is followed by hepatocellular carcinoma, Pikarsky et al. implicated TNFα upregulatoin
3 in tumor promotion of HCC, and suggest that TNFα and NF-κB are potential targets for cancer
4 prevention in the context of chronic inflammation. A similar conclusion was reached with
5 respect to NF-κB by an independent group of investigators using a model of experimental
6 dextran sulfate-induced colitis, in which inactivation of the IκB kinase resulted in reduced
7 colorectal tumors (Greten et al , 2004). Although there are many other clinical condition
8 supporting the concept of inflammation is a critical component of tumor progression (e.g., reflux
9 esophagitis/ esophageal cancer; inflammatory bowel disease/ colorectal cancer), there is at least
10 one notable example that does not fit this paradigm. As described below, psoriasis is a chronic
11 cutaneous inflammatory disease, which is seldom if ever accompanied by cancer suggesting the
12 relationship between tissue repair, inflammation, and development may not be as simple as
13 portrayed by the aforementioned reviews and experimental results. Besides psoriasis, other
14 noteworthy observations pointing to more complexity include the observation that in the Mdr2
15 knockout mice, we rarely detect bile duct tumors despite extensive inflammation, NF-κB
16 activation, and abundant proliferation of bile ducts in portal spaces (Pikarsky et al., 2004).
17 Moreover, in a skin cancer mouse model, NF-κB was shown to inhibit tumor formation (Dajee et
18 al., 2003). Thus, the composition of inflammatory mediators, or the properties of the responding
19 epithelial cells (e.g., signaling machinery, metabolic status), may dictate either tumor promotion
20 or tumor suppression. Chronic inflammation and tissue repair can trigger pro-oncogenic events,
21 but also that tumor suppressor pathways may be upregulated at various sites of injurty and
22 chronic cytokine networking."
23
24   "One cannot easiliy dismiss the many dilemmas raised by the psoriatic plaque that
25 confound a simple link between the tissue repair, inflammation, and carcinogenesis. Since it is
26 easily visible to the naked eye, and patients may suffer from such lesions for decades, it is
27 difficult to argue that various skin cancers such as squamous cell carcinoma, basal cell
28 carcinoma, or melanoma actually do develop within plaques by are being overlooked by patients
29 and dermatologists. Remarkably, psortiatic plaques are intentionally exposed to mutagenic
30 agents including excessive sunlight, topical administration of crude coal tar, or parenteral DNA
31 cross-linking agent –psoralen followed by ultraviolet light. Moreover these treatments are
32 known to induce skin cancer in nonlesional skin. Thus since psoriatic skin is characterized by
33 altered differentiation, angiogenesis, increased telomerase activity, proliferative changes, and
34 apoptosis resistance, one would expect that each and every psoriatic plaque would be converted
35 to cancer, or at least serve as fertile soil for the presence of non-epithelial skin cancers over
36 time." "In conclusion, it would seem prudent to remember the paradigm proposed by Weiss
37 (1971) in which he suggested that premalignant cells do not comprise an isolated island, but are a
38 focus of intense tissue interactions. The myriad inflammatory effects of the tumor
39 microenvironment are important for understanding tumor development, as well as tumor
40 suppression and senescence, and for the design for efficacious prevention strategies against
41 inflammation-associate cancer" (Nickoloff et al., 2005) .

3.3.4. Gender Influences on Susceptibility

As discussed previously, male humans and rodents are generally more likely to get HCC. The increased risk of liver tumors from estrogen supplements in women has been documented. In mice male TCE exposure has been shown to have greater variability in response and greater effects on body weight in males (Kjellstrand et al., 1983a, 1983b) but to also induce dose-related increases in liver weight and carcinogenic response in female mice as well as males (see Section 2.3.3.2). Recent studies have attempted to link differences in inflammatory cytokines and gender differences in susceptibility.

Lawrence et al. (2007) suggest that "studies of Naugler et al. (2007) and Rakoff-Nahoum and Medzhitov (2007), advance our understanding of the mechanisms of cancer-related inflammation. They describe an important role for an intracellular signaling protein called MyD88 in the development of experimental liver and colon cancers in mice. MyD88 function has been well characterized in the innate immune response (Akira and Takeda, 2004), relaying signals elicited by pathogen-associated molecules and by the inflammatory cytokine interleukin-1 (IL-1)." "The conclusion from Naugler et al. (2007) and Rakoff-Nahoun and Medzhitov is that MyD88 may function upstream of NF-κB in cells involved in inflammation-associated cancer. Immune cells infiltrate the microenvironment of a tumor. Naugler et al. (2007) and Rakoff-Nahoun and Medzhitov (2007) suggest that the development of liver and intestinal cancers in mice may depend on a signaling pathway in infiltrating immune cells that involved the protein MyD88, the transcription factor NF-κB, and the pro-inflammatory cytokine Il-6. TLR binds a ligand which acts on MyD88 which acts on NF-κB which leads to secretion of inflammatory cytokine IL-6 which leads to promotion of tumor cell survival and proliferation."

Naugler et al. (2007) suggested gender disparity in MyD88–dependent IL-6 production was linked to differences in cancer susceptibility using the DEN model (a mutagen with concurrent regenerative proliferation at a single high dose) with a single injection of DEN. Partial hepatectomy was reported to induce no gender-related difference in IL-6 increase. After DEN treatment the male mouse had 275 ng/ml as the peak IL-6 levels 12 hours after DEN and for female mice the peak was reported to be 100 ng/ml 12 hours after DEN administration. This is only about a 2.5 fold difference between genders. Il-6 mRNA induction was reported for mice 4 hours after DEN while at 4 hours, at a time when there was no difference in serum IL-6 between male and female mice. It was not established that the 4 hour results in mRNA translated to the differences in serum at 12 hour between the sexes. The magnitude of mRNA differences does not necessarily hold the same relationship as the magnitude in serum protein. In fact, there was not a linear correlation between mRNA induction and IL-6 serum levels.

A number of issues complicate the interpretation of the results of the study. The study examined an acute response for the chronic endpoint of cancer and may not explain the differences in gender susceptibility for agents that do not cause necrosis. The DEN was administered in 15-day old mice (which had not reached sexual maturity) for tumor information at a much lower dose than used in short-term studies of inflammation and liver injury in which

1   mature mice were used.   If large elevations of IL-6 are the reason for liver cancer, why doesn't a
2   partial hepatectomy induce liver cancer in itself? The percentage of proliferation at 36 and 48
3   hours after partial hepatectomy was the same between the sexes. If a 2.5-fold difference in IL-6
4   confers gender susceptibility, it should do so after partial hepatectomy and lead to cancer. For
5   female mice, partial hepatectomy showed alterations in a number of parameters. However,
6   partial hepatectomy does not cause cancer alone. The 5-fold increase 4 hours after DEN
7   induction of IL-6 mRNA in male mice is in sharp contrast to the 27 fold induction of IL-6 1 hour
8   after partial hepatectomy (in which at 4 hours the IL-6 had diminished to 6-fold). There
9   appeared to be variability between experiments (e.g., the difference in males between
10 experiments appears to be the same magnitude as the difference between male and female in one
11 experiment and the baseline of IL-6 mRNA induction appeared to be highly variable between
12 experiments as well as absolute units of ALT in serum 24 and 48 hours after DEN treatment that
13 tended to be greater that the effects of treatments) with experiments using very few animals
14 (n=3) for most treatment groups. Of note is that the MyD88 -/- male mice still had a background
15 level of necrosis similar to that of WT mice at 48 hours after DEN treatment a time, long after
16 the peak of IL-6 mRNA induction and IL-6 serum levels were reported to have peaked .One of
17 the key issues regarding this study is whether difference in IL-6 reported here lead to a in
18 proliferation and does that difference within 48 hours of a necrotizing dose of a carcinogen
19 change the susceptibility to cancer?   This report shows that male and female mice have a
20 difference in necrosis after CCL4 and a difference in proliferation. But are early differences in
21 IL-6 at 4 hours related to the same kind of stimulus that leads to necrosis and concurrent
22 proliferation?   The amount of proliferation between male and female mice 48 hours after DEN
23 was very small and the study was conducted in a very few mice (n = 3). At 36 hours the
24 proliferation was almost the same between the genders and about 0.6% of cells. The baseline of
25 proliferation also differed between genders but the variation and small number of animals made
26 it insignificant statistically. At 48 hours the differences in proliferation between male and female
27 mouse were more pronounced but still quite low (2% for males and ~ 1% for females).   Is the
28 change in proliferation just a change in damage by the agent? Given the large variation in serum
29 ALT and by inference necrosis, is there an equal amount of variability in proliferation? This
30 study gives only limited information for DEN treatment.
31
32   The difference in incidence of HCC was reported to be greater than that of proliferation
33 between genders and of other parameters although differences in tumor multiplicity or size
34 between the genders are never given in the paper. Most importantly, comparisons between the
35 short-term changes in cytokines and indices of acute damage are for adult animals that are
36 sexually mature and at doses that are 4 times (100 vs. 25 mg/kg) that of the sexually immature
37 animals who are going through a period of rapid hepatocyte proliferation (15 day old animals).
38 It is therefore difficult to extrapolate between the two paradigms to distinguish the effects of
39 hormones and gender on the response. Finally, the work of Rakoff-Hahoun and Medzhitov
40 (2007) showed that it is the effect of tumor progression and not initiation that is affected by
41 MyD88 (a signaling adaptor to Toll-like receptors). Thus looking at parameters at the initiation
42 phase at necrotic doses for liver tumors may not be relevant.

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1   **3.3.5. Epigenomic Modification**
2
3   There are several examples of chemical exposure to differing carcinogens that have lead
4   to progressive loss of DNA methylation (i.e., DNA hypomethylation) including TCE and its
5   metabolites. The evidence for TCE and its metabolites is specifically discussed in Section
6   3.4.2.2. below. Other examples of carcinogens that have been noted to change DNA methylation
7   are early stages of tumor development include ethionine feeding, phenobarbitol, arsenic,
8   dibromoacetic acid, and stress. However, it has not yet been established whether epigenetic
9   changes induced by carcinogens and found in tumors play a causative role in carcinogenesis or
10   are merely a consequence of the transformed state (Tryndyak et al., 2006). Tamoxifen has been
11   reported to be an example of a chemical that induced genetic and epigenetic changes (Tryndyak
12   et al., 2006).
13
14   Pogribny et al. (2007) report the effect of WY-14,643 on global mouse DNA
15   hypomethylation exposed at 1000 ppm for 1 week, 5 weeks or 5 months. What is of particular
16   note in this study is that at this exposure level, one commonly used for MOA studies using WY-
17   14,643 to characterize the effects of PPARα agonists as a class, there was significant
18   hepatonecrosis and mortality reported by Woods et al. (2007b). Both wild type and PPARα-/-
19   null mice were examined. In wild type mice DNA syntheses was elevated 3-, 13-, and 22-fold of
20   time-matched controls after 1 week, 5 weeks, and 5 months of WY 14,543 treatment. Changes in
21   ploidy were not examined. After 5 weeks of exposure, the ratio of unmethylated CpG cites in
22   whole liver DNA was the same for WY-14,643 treatment and control but by 5 months there was
23   an increase in hypomethylation in WY-14,643 treated wild type mice.   The authors did not
24   report whether foci were present or not which could have affected this result. The similarity in
25   hypomethylation at 5 days and 5 weeks, that also had a small probability of foci development, is
26   suggestive of foci affecting the result at 5 months. For PPAR -/- mice there was increased
27   hypomethylation reported at 1 week and 5 weeks after WY-14,643 treatment that was not
28   statistically significant with so few animals studied and at 5 months the null mice had decreased
29   hypomethylation compared to 1 and 5 weeks. The authors note that, methylation of c-Myc genes
30   was reported to not be affected by long-term dietary treatment with WY-14,643 even though
31   WY-14,643-related hypomethylation of c-Myc gene early after a single dose of WY-14,643 has
32   been observed (Ge et al., 2001a).The authors concluded "thus, alterations in the genome
33   methylation patterns with continuous exposure to non-genotoxic liver carcinogens, such as WY,
34   may not be confined to specific cell proliferation-related genes."
35
36   Pogribny et al. (2007) reported Histone H3 and H4 trimethylation status in wild type and
37   PPAR null mice to show a rapid and sustained loss of histone H3K9 and histone H4K20
38   trimethylation in wild type mice fed WY-14,643 from 1 week to 5 months. There was no
39   progressive loss in histone hypomethylation with the same amount of demethylation occurring at
40   5 days, 5 weeks, and 5 months in wild type mice fed WY. The change from control was ~ 60%
41   reduction. The control values with time were not reported and all controls were pooled to give
42   one value (n = 15).   For PPAR-/-l mice there was a slight decrease with WY-14,643 treatment (
43   ~ 15%) reported. In wild type mice, WY-14,643 treatment was reported to have no effect on the

1   major histone methyltransferase, Suv39h1, while expression of another (PRDM/Riz1) increased
2   significantly as early as on week of treatment and remained elevated for up to five months. The
3   effect on expression of Suv420h2 (responsible for histone H4K20 trimethylation) was more
4   gradual and the amounts of this protein in livers of mice fed Wy-14m643 were reported to be
5   lower than in control. The authors did not examine these parameters in the null mice so the
6   relationship of these effects to receptor activation cannot be determined. Pogribny et al. (2007)
7   report hypomethylation of retroelements (LTR IAP, LINE1 and LINE2 retrotransposons)
8   following long-term exposure to WY-14,643 which the authors concluded can have effects on
9   the stability of the genome. Again these results are for whole liver that may contain foci.
10   Nevertheless, these findings raise questions about other target organs and a more general
11   mechanism for WY-14,643 effects than a receptor mediated one. The lack of effects on c-Myc
12   and the irrelevance of the transient proliferation through it reported here gives more evidence of
13   the irrelevance of a MOA dependent on transient proliferation. The authors stated that studies
14   show that a sustained loss of DNA methylation in liver is an early and indispensable event in
15   hepatocarcinogenesis induced by long-term exposure of both genotoxic and non-genotoxic
16   carcinogens in rodents. Thus, this statement argues against making such a distinction in MOA
17   for "genotoxic" and "non-genotoxic" carcinogens. Finally, the use of a dose which Woods et al.
18   (2007b) demonstrate to have significant hepatonecrosis and mortality, limits the interpretation of
19   these results and their relevance to models of carcinogenesis without concurrent necrosis.
20
21   Strain sensitivity to hepatocarcinogenicity has been investigated in terms of short-term
22   changes in methylation. Bombail et al. (2004) reported that a tumour-inducing dose of
23   phenobarbital reduced the overall level of liver DNA methylation in a tumour-sensitive
24   (B6C3F1) mouse strain but that the same dose of phenobarbital did not alter global methylation
25   level in a more tumour-resistant strain (C57BL/6), although the compound increased hepatocyte
26   proliferation in both strains (Counts et al., 1996). Bombail et al reported that "In a similar study,
27   Watson and Goodman (2002) used a PCR-based technique to measure DNA methylation
28   changes specifically in GC-rich regions of the mouse genome." Watson and Goodman (2002)
29   found that, that in these areas of the genome, exposure to phenobarbital caused an increase in
30   methylation in dosed animals compared with control animals. Again, the change was more
31   pronounced in tumour-prone C3H/He and B6C3F1 strains than in the less sensitive C57BL/6
32   strain. They also reported increased DNA synthesis in C57BL/6 mice but decreased global
33   methylation in the B6C3F1 strain after PB administration 1-2 weeks. The lifetime spontaneous
34   tumor rates were reported to be less than 5% in C57BL/6 mice but up to 80% in C3H/He mice.
35   Counts et al. (1996) reported that cell proliferation and global hepatic methylation status in
36   relatively liver tumor susceptible B6C3F1 with relatively resistant C57BL6 mice following
37   exposure to PB and/or chlorine/methionine deficient (CMD) diet. Cell proliferation was reported
38   to be higher in C57BL6 mice while transient hypomethylation occurred to a greater extent in
39   B6C3F1 mice after phenobarbital treatment while dual administration of CMD and PB led to
40   enhance cell proliferation and greater global hypomethylation with similar trends in terms of
41   strain sensitivities as compared with either treatment alone (i.e., greater increase in cell
42   proliferation in C57Bl/6 and greater levels of hypomethylation in B6C3F1). Thus, the authors
43   concluded that B6C3F1 mice have relatively low capacity to maintain the nascent methylation

1   status of their hepatic DNA. However, on the whole, the control values for methylation for the
2   C57BL/6 mice appear to be slightly higher than the B6C3F1 mice. Thus, claims that the liver
3   tumor sensitive B6C3F1 had more global hypomethylation after a promoting stimulus which
4   could be related to tumor sensitivity, is tempered by the fact that resistant strain had a higher
5   control baseline of hypomethylation. The baseline level of LI or hepatocyte proliferation appears
6   to be slightly higher in the C57BL/6 mouse. In addition, the largest strain difference in
7   hypomethylation after a CMD diet was at week 12 (135% of control for the B6C3F1 strain and
8   151% of control for the C57BL/6 strain) and this pattern was opposite that for the 1 week time
9   point. Thus, the suggestion by Counts et al. (1996), that the inability to maintain methylation
10   status by the B6C3F1 strain, is also not supported by the data for CMD diet.
11

3.4.   Specific Hypothesis for MOA of TCE Hepatocarcinogenicity in Rodents

13

3.4.1.   PPARα Agonism as the MOA for Liver Tumor Induction – The State of the Hypothesis

16

17   PPARα receptor activation has been suggested to be the MOA for TCA liver tumor
18   induction and for TCE liver tumor induction to occur primarily as a result of the presence of its
19   metabolite TCA (NAS, 2006). However, as discussed previously (Section 2.1.10), TCE-induced
20   increases in liver weight have been reported in male and female mice that do not have a
21   functional PPARα receptor (Nakajima et al., 2000). The dose-response for TCE-induced liver
22   weight increases differs from that of TCA (see Section 2.4.2). The phenotype of the tumors
23   induced by TCE have been described to differ from those by TCA and to be more like those
24   occurring spontaneously in mice, those induced by DCA, or those resulting from a combination
25   of exposures to both DCA and TCA (see Section 2.4.4). As to whether TCA induces tumors are
26   induced through activation of the PPARα receptor, the tumor phenotype of TCA-induced mouse
27   liver tumors has been reported to have a pattern of H-ras mutation frequency that is opposite that
28   reported for other peroxisome proliferators (see Section 2.4.4.; Bull et al 2002; Stanley et al.,
29   1994; Fox et al., 1990; Hegi et al., 1993). While TCE, DCA, and TCA are weak peroxisome
30   proliferators, liver weight induction from exposure to these agents has not correlated with
31   increases in peroxisomal enzyme activity (e.g. PCO activity) or changes in peroxisomal number
32   or volume. However, liver weight induction from subchronic exposures appears to be a more
33   accurate predictor of carcinogenic response for DCA, TCA and TCE in mice (see Section 2.4.4).
34   The database for cancer induction in rats is much more limited than that of mice for
35   determination of a carcinogenic response to these chemicals in the liver and the nature of such a
36   response.
37

38   The MOA for peroxisome proliferators has been the subject of research and debate for
39   several decades. It has evolved from an "oxidative damage" due to increased peroxisomal
40   activity to a MOA framework example developed by Klaunig et al. (2003) that reported that
41   described causal inferences to be made for hepatocarcinogenesis after a chemical exposure was
42   shown to activate of the PPAR-α receptor with concurrent perturbation of cell proliferation and
43   apoptosis, and selective clonal expansion. Of note although inhibition of apoptosis was proposed

1 as part of the sequellae of PPARα activation, as noted in Section 2.4.1., no changes in apoptosis
2 in mice exposed to TCE have been reported with the exception of mild enhanced apoptosis at
3 1000 mg/kg/day dose but more importantly that for mice the rate of apoptosis decreases as mice
4 age and appear to be lower than that of rats. While DCA exposure has been noted to reduce
5 apoptosis, the significance of DCA-induced reduction in apoptosis, from a level that is already
6 inherently low in the mouse, for the MOA for induction of DCA-induce liver cancer is difficult
7 to discern.
8
9   Klaunig et al. based causal inferences on the attenuation of these events in PPAR-α-null
10 mice in response to the prototypical agonist WY-14,643 with a number of intermediary events
11 considered to be associative (*e.g.*, expression of peroxisomal and non-peroxisome genes,
12 peroxisome proliferation, inhibition of gap junction intracellular communication, hepatocyte
13 oxidative stress as well as Kupffer cell-mediated events). The data set for di(2-ethylhexyl)
14 phthalate (DEHP) was prominently featured as an example of "PPAR-α induced
15 hepatocarcinogenesis." For DEHP PPAR-α activation was described as the initial key event with
16 evidence lacking for a direct effect but supported primarily supported by evidence from PPAR-α-
17 knockout mice treated with WY-14,643. Klaunig et al. concluded that "…all the effects
18 observed are due only to the activation of this receptor and the downstream events resulting from
19 this activation and that no other modes of action are operant"
20
21   Although that PPARα receptor activation is the sole MOA for DEHP has been cited by
22 several reports (including IARC 2000), several articles have questioned the adequacy of this
23 proposed MOA (Melnick 2001; Melnick 2002; Melnick 2003; Melnick et al., 2007; FIFRA
24 2004; Caldwell and Keshava, 2006; Caldwell et al., 2008b; Keshava and Caldwell, 2006; and
25 Keshava et al., 2006). New information is now available that also questions several of the
26 assumptions inherent in the proposed MOA by Klaunig et al. and the dismissal of PPARα
27 agonists as posing a health risk to humans. Specific questions have been raised about the use of
28 WY-14,643 as a prototype for PPARα (especially at necrogenic doses) and use of the PPARα -/-
29 null mouse in abbreviated bioassays to determine carcinogenic hazard.
30
31 *3.4.1.1. Heterogeneity of PPARα agonist effects and inadequacy of WY-14,643 paradigm as*
32 *prototype for class*
33
34   Inferences regarding the carcinogenic risk posed to humans by PPARα agonists have
35 been based on limited epidemiology studies in humans that were not designed to detect such
36 effects. However, as noted by Nissen et al. (2007) the PPARα receptor is pleiotropic, highly
37 conserved, has "cross talk" with a number of other nuclear receptors, and plays a role in several
38 disease states. "The fibrate class of drugs, which are PPARα agonists intended to treat
39 dyslipidemia and hypercholesterolemia, have recently been associated with a number of serious
40 side effects." While these reports of clinical side effects are for acute or subchronic conditions
41 do not (and would not be expected to) be able to detect liver cancer from fibrate treatment, they
42 clearly demonstrate that compounds activating the PPAR receptors may produce a spectrum of
43 effects in humans and the difficulty in studying and predicting the effects from PPAR agonism.

1   Graham et al. (2004) recently reported significantly increased incidence of hospitalized
2   rhabdomyolysis in patients treated with fibrates both alone and in combination with statins.
3   Even though pharmaceutical companies have spent a great deal of effort to develop agonists
4   which are selective for desired effects, the pleiotropic nature of the receptor continues to be an
5   obstacle.
6
7   Also, fibrates, WY-14,643 and other PPARα agonists pan agonists for other PPARs.
8   Shearer and Hoekstra (2003) note that fibrates, including Fenofibrate, Clofibrate, Bezafibrate,
9   Ciprofibrate, Gemfibrozil, and Beclofibrate are all drugs that were discovered prior to the
10   cloning of PPARα and without knowledge of their mechanism of action but with optimization of
11   lipid lowering activity was carried out by administration of candidates to rodents. They report
12   that many PPARα ligands, including most of the common fibrate ligands, show only modest
13   selectivity over the other subtypes with, for example, fenofibric acid and WY-14,643 showing <
14   10 fold selectivity for activation of human PPARα compared to PPARγ and/or PPARδ. In human
15   receptor transactivation assays they report:
16
17   Human receptor transactivation assays of EC50:
18
19   WY-14,643 = 5.0 μm for PPARα, 60 μm for PPAR γ, 35 μm for PPARδ.
20   Clofibrate = 55 μm for PPARα, ~500 μm for PPAR γ, inactive at 100 μm for PPARδ
21   Fenofibrate = 30 μm for PPARα, 300 μm for PPAR γ, inactive at 100 μm for PPARδ
22   Bezafibrate = 50 μm for PPARα, 60 μm for PPAR γ, 20 μm for PPARδ.
23
24   *Murine receptor transactivation assay of EC50*:
25
26   WY = 0.63 μm for PPARα, 32 μm for PPAR γ, inactive at 100 μm for PPARδ
27   Clofibrate = 50 μm for PPARα, ~500 μm for PPAR γ, inactive at 100 μm for PPARδ
28   Fenofibrate = 18 μm for PPARα, 250 μm for PPAR γ, inactive at 100 μm for PPARδ
29   Bezafibrate = 90 μm for PPARα, 55 μm for PPAR γ, 110 μm for PPARδ.
30
31   Thus these data show the relative effective concentrations and "potency for PPAR
32   activity" of various agonists in humans and rodents, rodent and human responses may vary
33   depending on agonist, agonists vary in what they activate between the differing receptors and
34   that there is a great deal of transactivation of these drugs.
35
36   For fibrates specifically, a study by Nissen et al. (2007) reports that in current practice, 2
37   fibrates, Gemfibrozil and Fenobibrate, are still widely used to treat a constellation of lipid
38   abnormalities known as atherogenic dyslipidemia and note that currently available fibrates are
39   weak ligands for the PPARα receptor and may interact with other PPAR systems. They note that
40   the pharmaceutical industry has sought to develop new, more potent and selective agents within
41   this class but, most importantly, that none of the novel PPARα agonists has achieved regulatory
42   approval and that according to a former safety officer in the U.S. Food and Drug Administration
43   (FDA) (El-Hage, 2007) that more than 50 PPAR modulating agents have been discontinued due

1 to various types of toxicity (e.g., elevations in serum creatinine, rhabdomylosis, "multi-species,
2 multi-site increases in tumor with no safety margin for clinical exposures, and adverse
3 cardiovascular outcomes) but without scientific publications describing the reasons for
4 termination of the development programs. Nissen et al. report differences in effect between a
5 more highly selective and potent PPARα agonist and the less potent and specific one in humans.
6 They note "a recent large study of Fenofibrate in patients with diabetes showed no significant
7 reduction in morbidity but a trend toward increased all-cause mortality (Keech et al. 2005, 2006).
8 Whether this potential increase in mortality is derived from compound specific toxicity of
9 Fenofibrate or is an adverse effect of PPARα activation remains uncertain." In addition to the
10 lack of publication of effects from PPAR agonists in human trials in which toxicity can be
11 examined as noted by Nissen et al., the Keech study is illustrative of the problem in trying to
12 ascertain liver effects from fibrate treatment in humans as the focus of the outcomes was
13 coronary events in a study of 5 years duration in a older diabetic population. As stated above,
14 the challenges the pharmaceutical industry and the risk assessor face in determining the effects of
15 PPAR agonists is "that these compounds and drugs modulate the activity of a large number of
16 genes, some of which produce unknown effects." Nissen et al. further note that "Accordingly,
17 the beneficial effects of PPAR activation appear to be associated with a variety of untoward
18 effects which may include, oncogenesis, renal dysfunction, rhabdomylosis, and cardiovascular
19 toxicity. Recently, the FDA began requiring 2-year preclinical oncogenicity studies for all
20 PPAR-modulating agents prior to exposure of patients for durations of longer than 6 months (El-
21 Hage, 2007)." Thus, while existing evidence for liver cancer in humans is null rather than
22 negative, there remains a concern for oncogenicity and many obstacles for determining such
23 effects through human study. The heterogeneity in response to PPARα agonists and the
24 heterogeneity of effects they cause (Keshava and Caldwell, 2006) are evident from these reports.
25
26   Many studies have used the effects of WY-14,643 at a very high dose and extrapolated
27 those findings to PPARα agonists as a class. However, this diverse group of chemicals have
28 varying potencies and effects for the "key events" described by Klaunig et al. (2003) (Keshava
29 and Caldwell, 2006). The standard paradigm used with WY-14,643 to induced liver tumors in
30 all mice exposed by 1 year (an abbreviated bioassay), uses a large dose that has also has been
31 reported to produced liver necrosis, which can have an effect of cell proliferation and gene
32 expression patterns, and to also induce premature mortality (Woods et al., 2007b). As stated
33 above, WY- 14,643 also has a short peak of DNA synthesis that peaks after a few days of
34 exposure, recedes, and then unlike most PPARα agonists studied (e.g., Clofibrate, clofibric acid,
35 Nafenopin, Ciprofibrate, DEHP, DCA, TCA and LY-171883) has a sustained proliferation at the
36 doses studied (Tanaka et al., 1992; Barrass et al 1993; Marsman et al., 1992; Eacho et al., 1991;
37 Lake et al 1993; Yeldani et al., 1989; David et al 1999; Marsman et al., 1988; Carter et al., 1995;
38 Sanchez and Bull 1990). Clofibrate has been shown to have a decrease in proliferation gene
39 expression shortly after its peak (see Section 3.2.2). As shown in above, WY-14,643
40 hepatocellular increases in DNA synthesis did not appear to have a dose-response (see Section
41 3.4.2), only WY-14,643 had a sustained elevation of Nf-κB (gem and dibutyl phthalate did not)
42 (see Section 3.4.3.3), and the effects on DNA methylation occurred at 5 months and not earlier
43 time points (when Foci were probably present) and effects of histone trimethylation were

1   observed to be the same from 1 weeks to 5 months (see Section 3.4.5). Such effects on the
2   epigenome suggest other effects of WY, -14,643 other than receptor activation and are not
3   specific to just WY-14,643 and are found in a number of conditions leading to cancer and in
4   tumor progression (see Sections 3.2.1 and 3.2.7.).
5
6   In their study of PPARα-independent short-term production of reactive oxygen species
7   from induced by large concentrations of WY-14,643 and DEHP in the diet, Woods et al. (2007c)
8   examined short-term exposures to (0.6% w/w DEHP or 0.05% or 500 pm WY-14,643 for 3 days,
9   1 weeks or 3 weeks) and reported that WY-14,643 induced a dramatic increase in bile flow that
10 was not observed from DEHP exposure. By 1 week of exposure there was a 5% increase in bile
11 flow for DEHP treatment but a 240% increase in bile flow for WY-14,643 treatment and by 3
12 weeks the difference in bile volume between treated and control was 12% for DEHP and 1100%
13 for WY-14,643 treated animals. The level of radical production was reported to be comparable
14 at 2 hours (after injection of 1.2 g/kg) and 3 weeks (0.6% w/w in diet) of DEHP exposure (~ 3
15 fold) (number of animals unknown).   In this oxygen radical formation as measured by spin
16 trapping in the bile was reported to be decreased after 3 days of treatment after DEHP and WY-
17 14,643 treatment.   However the large changes in bile flow by WY-14,643 treatment limit the
18 interpretation of this data along with a small number of   animals examined in this study (e.g., 6
19 control and DEHP animals and 3 animals exposed to WY-14,643 at 3 days), a 30% variation in
20 % liver/body weight ratios between control groups, and the insensitivity of the technique. In an
21 earlier study oxidative stress appears to be correlated with neither cell proliferation nor
22 carcinogenic potency (Woods et al., 2006). Woods et al. (2006) reported WY-14,643Y or DEHP
23 to induce an increase in free radicals at 2 hrs, a decrease at 3 days then an increase at 3 weeks for
24 both. However, radical formation did not correlate with the proliferative response, as DEHP
25 fails to produce a sustained induction of proliferative response in rodent liver but WY-14,643
26 does, and both WY-14,643 and DEHP gave a similar pattern of radical formation that did not
27 vary much from controls which is in contrast to their carcinogenic potency.
28
29   Although assumed to be a reflection of cell proliferation in many studies of WY-14,643
30 and by Klaunig et al. (2003), DNA synthesis recorded using the standard exposure paradigm for
31 WY-14,643, can also be a reflection of hepatocyte, nonparenchymal cell or inflammatory cell
32 mitogenesis (in the case of necrosis induced inflammation), from changes in hepatocyte ploidy,
33 or a combination of all. Other peroxisome proliferators have been shown to have a decrease in
34 proliferation gene expression shortly after their peaks (e.g., Clofibrate, see Section 3.2.2) and
35 both Methylclofenapate and Nafenopin have been shown to increase cell ploidy with Nafenopin
36 having the majority of its DNA synthesis a reflection of increased ploidy with only a small
37 percentage as increases in cell number (Section 3.4.1.). Several authors have also noted
38 increases in ploidy for WY-14,643 (see Section 3.4.1).
39
40   The Tg.AC genetically modified mouse was used to study 14 chemicals administered by
41 the topical and oral (gavage and/or diet) routes by Eastin et al. (2001). Clofibrate was considered
42 clearly positive in the topical studies but not WY-14,643 regardless of route of administration.
43 Based on the observed responses it was concluded by the workgroup (Assay Working Groups)

1   that the Tg.AC model was not overly sensitive and possesses utility as an adjunct to the battery
2   of toxicity studies used to establish human carcinogenic risk. The difference in result between
3   Clofibrate and WY-14,643 is indicative of a different MOA for the two compounds.
4
5   Similarly, at large exposure concentrations Boerrigter (2004) investigated the response of
6   male and female lacZ-plasmid transgenic mice treated at 4 months of age with 6 doses of 2,333
7   mg/kg DEHP, 200 mg/kg WY-14,643 or 90 mg/kg Clofibrate over a two week period mutation
8   frequencies assayed at 21 days following the last exposure. DEHP and WY-14,643 were shown
9   to significantly elevate the mutant frequency in both male and female liver DNA while
10   Clofibrate, as the dose level studied, was apparently non-mutagenic in male and female liver
11   (i.e., six-dose exposure to DEHP or WY-14,643 over a two week period significantly increased
12   the mutant frequency in liver of both female and male mice by approximately 40%). The author
13   notes that "the laxZ plasmid-based transgenic mouse mutation assay is somewhat unique among
14   other commercially available models (e.g. mutamouse and big blue), by virtue of its ability to
15   accurately quantify both point mutations and large deletions including those which originate in
16   the lacZ plasmid catamer and extend into the 3' flanking genomic region. It should be noted that
17   to date there is no single, agreed upon protocol for conducting mutagenicity assays with
18   transgenic rodents although several aspects have been upon by the Transgenic Mutation Assays
19   workgroup of the International Workshop on Genotoxicity Procedures."
20
21   For several chemicals both rats and mice demonstrate evidence of receptor activation
22   through peroxisome proliferation and peroxisome-related gene expression but only one develops
23   cancer. The herbicide, 2,4- dichlorophenoxyacetic acid (2,4-D), is a striking example of the
24   problems that would be associated with only using evidence of PPARα receptor activation to
25   make conclusions about MOA of liver tumors. 2,4-D is structurally similar to the PPARα
26   agonist Clofibrate and has been shown at similar concentrations to increase peroxisome number
27   and size, increase hepatic carnitine acetyltransferase activity and catalase, and decrease serum
28   triglycerides and cholesterol in rats (Vainio et al., 1983). Peroxisome number was also increased
29   in Chinese hamsters to a similar level as with Clofibrate at the same exposure concentration after
30   9 days of exposure to 2,4-D (Vainio et al., 1982).   In mice, Lundgren et al. (1987) report that
31   2,4-D exposure statistically increased the liver-somatic index over controls after a few days
32   exposure and increased mitochondrial protein, microsomal protein, carnitine acetyltransferase,
33   PCO activity, cytochrome oxidase, cytosolic epoxide hydrolase, microsomal epoxide hydrolase,
34   microsomal P450 content, and hepatic cytosolic epoxide hydrolase in mouse liver. Thus, 2,4-D
35   activates the PPARα receptor, with associated changes in peroxisome-related gene expression, in
36   multiple species and at similar doses to Clofibrate.. However, Charles et al. (1996) and Charles
37   and Leeming (1998) report that in several 2-year studies that there were no 2,4-D-induced
38   increases in liver tumors in F344 rats, CD1 rats, B6C3F1 mice and CD-1 mice. Another
39   example, is provided by Gemfibrozil, known as (5-2[2,5-dimethylphenoxy]2-2-
40   dimethylpentanoic acid) and [2,2-dimethyl-5-(2,5-xylyoxy) valeric acid], a therapeutic agent
41   that activates the PPARα receptor and is a peroxisome proliferator, but is carcinogenic only in
42   male rats but not female rats, nor in either gender of mouse (Contrera et al., 1997). Gemfibrozil
43   causes tumors in pancreas, liver, adrenal, and testes of male rats and causes increases in absolute

1 and relative liver weights in both rats and mice (Fitzgerald et al., 1981). Gemfibrozil, is a highly
2 effective lipid and cholesterol lowering drugs in humans and in mice (Olivier et al 1988).
3 However, although Gemfibrozil activates the PPARα receptor and induces peroxisome
4 proliferation in mice, it does not induce liver tumors in that species. In the longterm study of
5 Bezafibrate, Hays et al (2005) note that the role of this receptor in hepatocarcinogenesis has only
6 been examined using one relatively specific PPARα agonist (WY-14,643) and report that
7 Bezafibrate can induce the expression of a number of PPARα target genes (acyl CoA oxidase
8 and CYP4a) and increased liver weight in PPARα knockout mice that is not dependent on
9 activation of PPARβ or PPARγ. As noted by Boerrigter (2004), "In contrast to DEHP and WY-
10 14,643, Clofibrate produced hepatocellular carcinomas in rats only while no increase in the
11 incidence of tumors was reported in mice (Gold and Zeiger 1997). However, Clofibrate induces
12 peroxisome proliferation in both rats and mice (Lundgren and DePierre 1989) but only produced
13 hepatocellular carcinomas in rats (Gold and Zeiger, 1997)." Melnick et al. (1996) note that
14 similar levels of peroxisomal induction were observed in rats exposed to DEHP and DEHA
15 [di(2-ethylhexyl) adipate] at doses comparable to those used in the bioassays of these chemicals.
16 However, DEHP but not DEHA gave a positive liver tumor response in 2-year studies in rats. In
17 an evaluation of the carcinogenicity of tetrachloroethylene, an expert panel of the IARC
18 concluded that the weak induction of peroxisome proliferation by this chemical in mice was not
19 sufficient to explain the high incidence of liver tumors observed in an inhalation bioassay.
20
21   In adult animals, apoptosis acts as a safeguard to prevent cells with damaged DNA from
22 progressing to tumor, but like cell proliferation, alterations in apoptosis are common to many
23 MOAs. In addition, only short term data is available on changes in apoptosis due to PPARα
24 agonists, and long-term changes have not been investigated (Rusyn et al., 2006). For example,
25 although a decrease in apoptosis has also suggested to be an important additional molecular
26 event that may affect the number of cells in rodent liver following exposure to the peroxisome
27 proliferator DEHP, apoptosis rates have not investigated past 4 days of exposure and thus the
28 time-course of this event is uncertain. The antiapoptotic effects of PPAR agonists appear to also
29 be dependent on non-parenchymal cells (i.e., Kupffer cells) which do not express PPARα and
30 could be a transient event (Rusyn et al., 2006).   Morimura et al. (2006) report evidence for
31 exposure to WY-14,643 that does not support a role for PPARα –mediated apoptosis in tumor
32 formation (see Section 3.5.1.3., below) as well as appearing to be specific to WY-14,643 (see
33 Section 3.4.3.3.).
34
35   The lack of a causal relationship of transient DNA synthesis increases and
36 hepatocarcinogenesis has been raised by many (Caldwell et al 2008b) and is discussed in Section
37 3.4.2. as well as the changes in ploidy (see Section 3.4.1). In regard to gene expression profiles,
38 many studies have focused on gene profiles during the early transient proliferative phase or have
39 identified genes primarily associated with peroxisome proliferation as "characteristic" or relevant
40 to those associated with tumor induction. Several have focused on the number of genes whose
41 expression "goes up" or "goes down" from a small number of animals. Caldwell and Keshava
42 (2006) presented information on WY-14,643, dibutyl phthalate, Gemfibrozil and DEHP, and
43 noted inconsistent results between PPARα agonists, paradoxes between mRNA and protein

1 expression, strain, gender, and species differences in response to the same chemical, and time-
2 dependent differences in response for several enzymes and glutathione.
3
4 *3.4.1.2. New information on causality and sufficiency for PPARα receptor activation*
5
6   In its review of the EPA's draft risk assessment of perflurooctanoic acid (PFOA), the
7 Science Advisory Panel (FIFRA 2004) expressed concerns about whether PPARα agonism
8 constitutes the sole MOA for PFOA effects in the liver and the relevance to exposed fetuses,
9 infants, and children. In part based on uncertainties regarding the Klaunig et al. (2003) proposed
10 MOA, they concluded that the tumors induced by PFOA were relevant to human risk assessment.
11 The hypothesis that activation of the PPARα receptor is the sole mode of action
12 hepatocarcinogenesis induced by DEHP and many other chemicals is further called into question
13 by recent studies. In the case of DEHP, Klaunig et al (2003) assumed that WY-14,643 and
14 DEHP would operate through the same key events and that long-term bioassays of DEHP in
15 PPARα -/- knockout mice would be negative and hence demonstrate the need for receptor
16 activation for hepatocarcinogenesis from DEHP.
17
18   The fallacy of these assumption are illustrated by the recent report of the first 2-year
19 bioassay of DEHP in PPARα -/- knockout mice (Sv/129 background strain) that reported DEHP-
20 induced hepatocarcinogenesis (Ito et al., 2007). Similar to other studies, the PPAR-/- mice had
21 slightly increased liver weights in comparison to controls and treated wild type mice (~ 12%
22 increase over controls). In fact statistical analysis of the incidence data shows that adenomas
23 were significantly increased in PPARα -/- mice compared with wild type mice exposed to 500
24 ppm DEHP and that a significant dose-response trend for adenomas and adenomas plus
25 carcinomas was observed in PPARα -/- mice. Overall, the cancer incidences were consistent
26 with a previous study of DEHP (David et al., 1999) in B6C3F1 mice at the same doses for nearly
27 the same exposure duration. A strength of this study is that it was conducted at much lower
28 more environmentally relevant doses that did not significantly increase liver enzymes as
29 indications of toxicity. As noted by the authors, DEHP was reported to also induce
30 glomerularnephritis more often in PPARα-null mice because of the absence of PPARα-
31 dependent anti-inflammatory effect of antagonizing the oxidative stress and NF-κB pathway
32 (Kamijo et al., 2007). Thus, these data support that hypothesis that there is no difference in liver
33 tumor incidences between PPARα -/- mice and wild type mice in a standard non-abbreviated
34 exposure bioassay that does not exceed the maximal tolerated doses and that DEHP can induce
35 hepatotoxicity as well as other effects independent of action of the PPARα receptor.

E-2
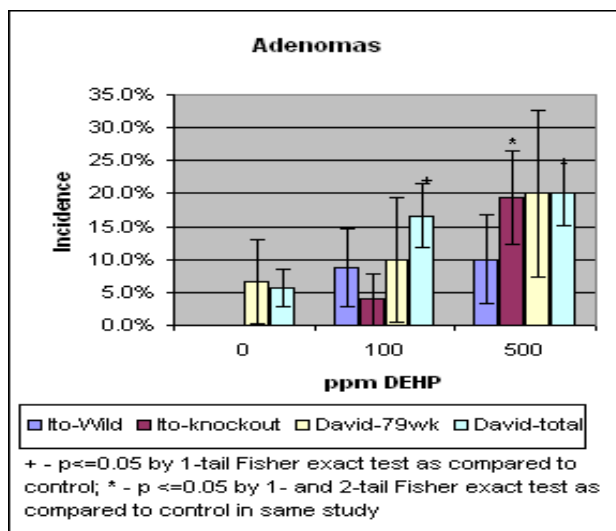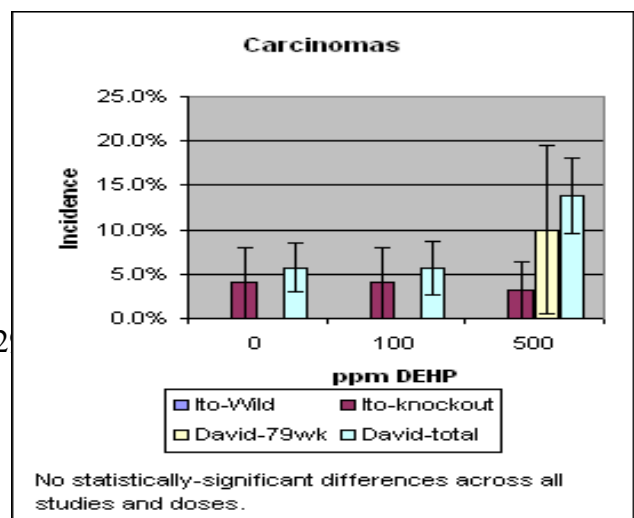
1   The study of Yang et al. (2007a) informs as to the sufficiency of PPARα receptor
2   activation and subsequent molecular event for hepatocarcinogenesis in mice. The study used a
3   VP16PPARα transgene under control of the liver-enriched activator protein (LAP) promoter to
4   constituitively activate the PPARα receptor in mouse hepatocytes. LAP- VP16PPARα
5   transgenic mice showed a number of effects associated with PPARα receptor activation
6   including decreased serum triglycerides and free fatty acids, peroxisome proliferation, enhanced
7   hepatocyte DNA synthesis and induction of cell-cycle genes and those described as "PPARα
8   targets" to comparable levels reported for WY-14,643 exposure. Hepatocyte proliferation as
9   determined the labeling index into hepatocyte nuclei was increased after 2 weeks of WY-14,643
10   treatment over controls (20.5% vs. 1.6% in control livers) with the LAPVP16 PPARα mice
11   giving a similar results (20.8% vs. 1.0% in control livers). The authors note that transgenic mice
12   did not appear to have positive labeling of nonparenchymal cell nuclei that were present in the
13   WY-14,643 treated animals. The transferase-mediated dUTP nick end-labeling assay results
14   were reported to show that there was no difference in apoptosis in wild type mice treated with
15   WY-14,643, the transgenic mice or and controls. In a small number of animals, microsomal
16   genes (CYP4A), peroxisomal (ACOX, BIEN – the bifunctional enzyme) and mitochondrial fatty
17   oxidation genes (LCAD – long chain acyl CoA dehydrogenase and VLCAD – very long chain
18   acyl CoA dehydrogenase) were expressed in the transgenic mice with WY-14,643 also
19   increasing expression of these genes in wild type mice but with less LPL than the transgenic
20   mice. Hepatic coA oxidation, were increased to a similar level in wild type mice treated with
21   WY-14,643 and the transgenic mice (n = 3-4) and were statistically different than controls. LAP-
22   VP16PPARα transgenic mice (8 weeks of age) exhibited hepatomegaly (~ 50 increase %
23   body/liver weight over controls), and an accumulation of lipid due to triglycerides but not
24   cholesterol. However, compared to wild-type mice exposed to WY-14,643 for two weeks, the
25   extent of hepatomegaly was reduced (i.e., % liver/body weight increase of ~ 2.5 fold with WY-
26   14,643 treatment), no hepatocellular hypertrophy or eosinophilic cytoplasms and no evidence of
27   non-parenchymal cell proliferation were observed in the LAP- VP16PPARα transgenic mice.
28
29   At ~1 year of age, Yang et al (2007a) reported there to be no evidence of preneoplastic
30   lesions or hepatocellular neoplasia in LAP- VP16PPARα transgenic mice, in contrast to 11
31   months of exposure to WY-14,643 in wild type mice. Microscopic examination of liver sections
32   were consistent with the gross findings, as hepatocellular carcinomas and hepatic lesions were
33   observed in the long-term WY-14,643 treated wild type mice, but not in > 20 LAP-VP16 PPARα
34   mice at the age of over 1 year in the absence of dox. There was no quantitative information on
35   tumors given nor of foci development in the WY-14,643 mice. As noted by Yang et al. (2007a)
36   PPARα activation only in mouse hepatocytes is sufficient to induce peroxisome proliferation and
37   increased DNA synthesis but not to induce liver tumors. Thus, "hepatocyte proliferation"
38   indentified by Klaunig et al. (2003) as a "causal event" in their PPARα MOA is not sufficient to
39   induce hepatocarcinogenesis. These data not only call into question the adequacy of the MOA
40   hypothesis proposed by Klaunig et al. (2003), but suggest only multiple mechanisms and also
41   multiple cell types may be involved in hepatocarcinogenicity caused by chemicals that are also
42   PPARα agonists.

1 *3.4.1.3 Use of the PPAR-/- knockout and humanized mouse*
2
3   Great importance has been attached to the results reported for PPARα -/- mice and their
4 humanized counterpart with respect to inferences regarding the MOA or peroxisome
5 proliferators and whether short-term chemical exposures or abbreviated bioassays conducted
6 with these mice can show that a PPARα MOA is involved. Consequently, the use of these
7 models warrants scrutiny. Compared to untreated wild type mice, liver weights in knockout
8 mice or humanized mice have been reported to be elevated (Voss et al., 2006; Laughter et al.,
9 2004; Morimura et al., 2006) and within 10% of each other (Peters et al.,.1997). In order to be
10 able to assign affects to a test chemical tested in knockout mice, a better characterization is
11 needed of the baseline differences between PPARα -/- knockout and wild-type mice. This is
12 particularly important for examining weak agonists because the changed they induce may be
13 small and need to be confidently distinguished from differences due to the loss of the receptor
14 alone. As shown by the Ito et al. (2007) study and as noted by Maronpot et al. (2004), there is a
15 need for lifetime studies to characterize background or spontaneous tumor patterns and life spans
16 (including those of the background strain). While the original work by Lee et al. (1995)
17 describes "the mice homozygous for the mutation were viable, healthy, and fertile and appeared
18 normal," the authors did not describe the survival curves for this model nor their background
19 tumor rate. In fact, further work has shown that they carry a background of chronic conditions,
20 including: (1) chronic diseases such as obesity and steatosis (Akiyama et al., 2001; Costet et al.,
21 1998); (2) altered hepatic of hepatocellular structure and function, such as vacuolated
22 hepatocytes (Voss et al., 2006, Anderson et al., 2004), also seen in "humanized" mice (Cheung et
23 al., 2004); and (3) altered lipid metabolism, including reduced glycogen stores, blunted hepatic
24 and cardiac fatty acid oxidation enzyme system response to fasting, elevated plasma free fatty
25 acids, fatty liver (steatosis), impaired gluconeogenesis, and significant hepatic insulin resistance
26 (Lewitt el al., 2001). Howroyd et al. (2004) reported decreased longevity and enhancement of
27 age-dependent lesions in PPARα -/- mice.
28
29   These baseline differences from wild-type mice may render them more susceptible to
30 toxic responses or shorten their lifespans with chemical exposure. For example, after
31 administration of 250 microliters CCL4/kg, all male and 40% of female PPARα knockout mice
32 were dead or moribund after 2 days of treatment, whereas 25 % of male wild-type mice and none
33 of the female wild-type mice exhibited outward signs of toxicity (Anderson et al., 2004). Hays
34 et al. (2005) reported that 100% of PPARα knockout have cholestasis after 1 year of Bezafibrate
35 treatment with higher bile acid concentration than wild-type mice. Lewitt et al (2001) noted that
36 male knockout mice have more marked accumulation of hepatic fat, hypercholesterolemia and to
37 be particularly sensitive to fasting with some dying if fasted for more than 24 hours. Sexual
38 dimorphism but especially increased susceptibility of the male mouse has been reported for
39 knockout mice with pure Sv/129 backgrounds (Lewitt et al., 2001; Anderson et al 2004) as well
40 as those with a suggested C57BL/6N background (Djouadi et al., 1998, Costet et al., 1998).
41 Akiyama et al. (2001) showed an apparent greater sexual dimorphism in mice with a pure Sv/129
42 background than C57BL/6N in regard to weight gain from 2 to 9 months but not in changes in
43 body weight or liver weight between wild-type and knockout animals. Adipose tissue, serum

1   triglycerides and cholesterol were altered in the knockout animals. Given that the experiment
2   was only carried out for 9 months, changes in body fat, liver weight and lipid levels may be
3   greater as the animals get older and steatosis is more prevalent. The dramatic effect on survival
4   as well as gender difference by the increased expression of lipoprotein lipase in the PPARα
5   knockout mouse with further genetic modification is demonstrated by Nohammer et al (2003)
6   who reported 50% mortality in 6 months and 100% mortality within 11 months of age while
7   females survived. These differences could affect the results of tumor induction for PPARα
8   agonists with less potency than WY-14,643 that do not produce tumors so rapidly. In addition
9   these studies suggest the need for careful consideration of the effects of use of different
10 background strains for the knockout and the need for careful characterization of the background
11 responses of the mouse model and the effects of the use of different background strains for the
12 knockout. Morimura et al. (2006) reported that, using the B6 background strain, there were only
13 foci at time periods but knockouts with the SV129 background had multiple tumors after WY-
14 14,643 treatment.
15
16   PPARα knockout mice have also been used to examine the dependence on PPARα on
17 changes in cell signaling, protein production, or liver weight. However, to be useful, the changes
18 incurred just by loss of the PPARα should also be well described. Reported differenced between
19 PPARα-knockout and wild-type mice can impact the sensitivity and specificity of these markers
20 of for the hypothesized MOA. In regards to altered cell signaling, Wheeler et al. (2003) note that
21 in normal cells p21^{waf} and p27^{kip1} inhibit the Cdk/cyclin complexes responsible for cell cycle
22 progression through G1/S transition. While these cellular signaling molecules are down-
23 regulated in response to partial hepatectomy in normal mice, they remain elevated in PPARα
24 knockout mice along with decreased DNA synthesis. Fumonisins are hepatocarcinogens that
25 have been associated changes in apoptosis and tissue generation, and increased acyl-CoA
26 oxidase and CYP4A (markers of PPARα activation) (Martinez-Larranaga et al., 1996). Voss et
27 al. (2006) report that the average number of hepatic apoptotic foci per mouse induced by
28 Fumonisins were 3-fold higher and liver mitotic figures counts were 2-fold lower in PPARα
29 knockout in comparison to wild-type mice, thus illustrating a difference in proliferative response
30 in the mice. PPARα-null mice have increased apoptosis and decreased mitosis with fumonisin
31 treatment. Voss et al. (2006) also report several differences in gene expression in wild-type and
32 PPARα knockout mice that ranged from 0.3% to 483% of the activity of wild-type mice. The
33 complex expression patterns of gene expression and determining their mechanistic implications
34 in regard to hepatotoxicity and carcinogenicity are difficult. Certainly the vast number of genes
35 whose expression is affected by WY-14,643 (1012 genes as cited by Voss et al., 2006) illustrates
36 such complexity. Voss et al. (2006) conclude that studies should consider dose- and time course-
37 related effect as well as species and strain-related differences in the expression of gene products.
38
39   The "humanized" PPARα mouse has a human copy of PPARα inserted into a PPARα
40 knockout mouse. It is inserted in a tetracycline response system so that in the absence of DOX
41 only human PPAR alpha is transcribed in humanized mouse liver and not in other tissues. A
42 rigorous examination of newly emerging studies regarding the "humanized" mouse is warranted.
43 There are two papers that have been published using the humanized PPARα mouse (Cheung et

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al., 2004; Morimura et al., 2006). Many of the issues described above for PPARα -/- mice are of concerned for the humanized knockout mouse. In addition, the placement of the humanized PPAR gene is a potential confounding factor, as discussed by Morimura et al. (2006): "It also cannot be ruled out that the hPPARα mice are resistant to the hepatotoxic effects of peroxisome proliferators due to the site of expression of the human receptor. The cDNA was placed under control of the tetracycline regulatory system and the liver-specific Cebp/B promoter that is preferentially expressed in hepatocytes."

In the Cheung et al. (2004) report, the humanized mouse was fed WY-14,643 for 2 or 8 weeks (age not given for the mice). WY-14,643 and Fenobrate were reported to decrease serum total triglyceride levels in wild and humanized mice to about the level seen in PPARα -/- mice (which were already suppressed without treatment).   Hepatomegaly and increase in hepatocyte size were observed in the PPARα -humanized mice fed WY-14,643 for 2 weeks but less than wild mice. By contrast, Morimura et al., (2006) state that the humanized mice did not exhibit hepatomegaly after treatment with WY-14,643. Cheung et al (2004) present figures that show increased vacuolization of hepatocytes in a control humanized mouse in comparison to wild type mice. Vacuolization increased with WY-14,643 treatment in the humanized mouse. Therefore there was a background level of liver dysfunction in these mice even with humanized PPARα. Vacuolization is consistent with fatty liver observed in the non-humanized PPARα-/- mouse. The authors reported that the humanized mouse did not have increased #s of peroxisomes after WY treatment. However, they present a figure for genes encoding peroxisomal , mitochondrial, and microsomal fatty acid oxidation enzymes that shows they were still markedly increased in PPARα -humanized mice following 8 weeks of exposure to WY-14,643. Therefore there is a paradox in these reported results.

Morimura et al. (2006) provided a useful example to illustrate the many issues associated with interpreting studies with genetically-altered animals. While this study is suggestive of a difference in susceptibility to tumor induction between wild-type and PPARα humanized mice, a conclusion that human PPARα is refractory to liver tumor induction is not sufficiently supported by this study. This study had uneven durations of exposure and follow-up and reported substantial toxicity or mortality that limit the interpretation of the observed tumor rates. For example, the 6 week-old male "humanized" mice had a 44-week experimental period but for "wild-type" mice that period was 38 weeks. In addition, for humanized mice, 10 mice were treated with 0.1% WY-14,643 with 20 controls, but for wild-type mice, 9 mice were given 0.1% WY with 10 controls. Furthermore, wild-type, WY-14,643-treated animals has suppressed growth and only a 50% survival to 38 weeks, so an effective LD50 has been used for this length of exposure. Specifically, of the 10 wild-type WY-14,643 treated mice, 3 died of toxicity and 2 were killed due to morbidity and their tissues examined. Humanized mice had a similar growth for animals treated with WY-14,643 and controls with only one mouse killed because of morbidity. Therefore the reported results, including tumor numbers, are for a mixture of different exposure durations and ages of animals. In addition the results of the study were reported for only on exposure level.

Furthermore, it is interesting that while control humanized mice had no adenomas, WY-14,643 treated humanized mice had one. Morimura et al. (2006) noted that this adenoma had a morphology "similar to spontaneous mouse liver tumor with basophilic and clear hepatocytes," whereas the tumors in wild-type mice treated with WY-14,643 were more diffusely basophilic. If the humanized animals were allowed to liver their natural lifespan, this raises the possibility that WY-14,643 may induce tumors that are similar to other carcinogens rather than those which have been characterized as "characteristic" of peroxisome proliferators (see Section 3.5.1.5) when human PPARα is present. Therefore the humanized PPARα rather than mouse PPARα may have an association with a tumor phenotype characteristic of other MOAs but this study need to be carried out for a longer period of exposure and with more animals to make that determination. The baseline tumor response of PPARα humanized mice needs to be characterized as well as tumors exposure to WY-14,643 or other carcinogens acting through differing MOAs. The numbers of foci were not reported, but "altered foci" were detected in one humanized mouse with WY-14,643 treatment and one without treatment. The phenotypes of the foci were not given by the authors

As discussed above, changes in liver weights have been used as a marker for susceptibility to liver tumor induction and the issues regarding baseline differences in PPARα -/- mice are equally relevant for PPARα humanized mice. Morimura et al. (2006) reported that absolute liver weight for control humanized mice at 44 weeks was 1.57 g (n=10). The absolute liver weight for wild control mice was 1.1 g (n=9) at 38 weeks. The final body weights differed by 14% but liver weights differed by 30%. Therefore, even though comparing different aged mice, the control humanized mice had greater liver size than the wild type control mice on an absolute and relative basis. This is consistent with humanized knockout mice having greater sized livers and a baseline of hepatomegaly. With treatment, Morimura et al. (2006) report that PPARα humanized mice treated with WY-14,643 had greater absolute and relative liver weights than controls but less elevations than wild-type treated animals. However, because half of the wild-type animals died, it is difficult to discern if liver weights were reported for moribund animals sacrificed as well as animals that survived to 38 weeks for wildtype mice treated with WY-14,643. However, it appears that moribund animals were included that were sacrificed early for treated groups and that values from the animal killed at 27 weeks were added in with those surviving till 45 weeks in the PPARα humanized mice treated with WY-14,643 group.

With respect to the gene expression results reported by Morimura et al. (2006), it is important to note that they are for liver homogenates with a significant portion of the nuclei from nonparenchymal cell of the liver (e.g., Kupffer and stellate cells). Thus, the results represent changes resulting from a mixture of cell types and from differing zones of the liver lobule, with potentially different gene changes merged together. Livers without macroscopic nodules were used for western blot and but could have contained small foci in the homogenate as well. The gene expression results were also reported for an exposure level of WY-14,643 that is an LD50 in wild type mice and could reflect toxicity responses rather than carcinogenic ones. The samples were also obtained at the end of the experiment (with a mix of durations of exposure) and may not reflect key events in the causation of the cancer but events that are downstream.

1   These limitations notwithstanding, it is interesting that expression of p53 gene was
2   reported by Morimura et al. (2006) to be increased in PPARα humanized mice treated with WY-
3   14,643 compared to all other groups. Furthermore, of the cell cycle genes that were tested, (i.e.,
4   *CD1*, *Cyclin-dependent Kinases 1 and 4*, and *c-myc*) there was a slightly greater level of *c-myc*
5   and *CD1* in control PPARα humanized mice than control wild type mice as a baseline. This
6   could indicate that there was already increased cell cycling going on in the control PPARα
7   humanized mouse and could be related to the increased liver size. Treatment with WY-14,643
8   induced an increase in cycling genes in wild-type mice in relation to its control, but whether that
9   induction was greater than control levels for PPARα humanized mice for *c-myc* and *CDk4* was
10   not reported by the authors. Apoptosis genes were reported to have little difference between
11   control PPARα humanized and wild type mice but to have a greater response induced by WY-
12   14,643 in humanized mice for *p53* and *p21*. There was no consistent or large change in
13   apoptosis genes in response to exposure to WY-14,643 in wild type mice. The increased
14   response of apoptosis genes in PPARα humanized mice without corresponding tumor formation
15   does not support that response as a key event in the MOA (neither does the lack of response from
16   WY-14,643 in wild type mice). For genes associated with PPARα peroxisomal (Acox),
17   microsomal (CYP4a) mitochondrial fatty oxidation (Mcad) and especially malic enzyme, there
18   was a greater response in wild type than PPARα humanized mouse after treatment with WY-
19   14,643. However, this is somewhat in contrast to Cheung et al. (2004), who reported increased
20   in some genes encoding peroxisomal, mitochondrial, and microsomal fatty oxidation enzymes in
21   the PPARα humanized mouse after treatment with WY-14,643.

22

23   The results reported by Yang et al. (2007b) use another type of "humanized" mouse to
24   study PPARα effects. Yang et al. (2007b) used a PPARα humanized transgenic mouse on a
25   PPAR-/- background that has the complete human PPARα (hPPARα) gene on a PAC genomic
26   clone, introduced onto the mouse *ppara*-null background and express human PPARα not only in
27   the liver but also in other tissues. Mice were administered WY-14,643 or Fenofibrate [0.1% or
28   0.2% (w/w)]. The authors show a figure representing expression of the hPPARα for two mice
29   with the tissue used for the genotyping with great variation in expression between the two cloned
30   mice indicated by intensity of staining. The authors state that in agreement with RNA
31   expression, hPPARα protein was highly expressed in the liver of hPPARα^{PAC} mice to an extent
32   similar to the mPPARα in wild-type mice. They report that following two weeks of Fenofibrate
33   treatment, a robust induction of mRNA expression of genes encoding enzymes responsible for
34   peroxisomal (ACOX), mitochondrial (MCAD,LCAD), microsomal (CYP4A) and cytosolic
35   (ACOT) fatty acid metabolism were found in liver, kidney and heart of both wild-type and
36   hPPARα^{PAC} mice indicating that hPPARα functions in the same manner as mPPARα to regulate
37   fatty acid metabolism and associated genes. However, the authors did no measure in Fenofibrate
38   treated animals, only WY-14,643 raising the issue of whether there a difference in the relative
39   mRNA expression of genes for ACOX etc and lipids between the two peroxisomal proliferator
40   treatments. The expression of enzymes associated with PPAR induction was presented only for
41   mice treated with Fenofibrate. However, the lipids results were presented only for mice treated
42   with WY-14,643. Therefore it cannot be established that these two agonists give the same
43   response for both parameters. Also for the enzymes the relative expressions to wild type controls,

1   the absolute expression and its variation between animals is not reported. It appears that the
2   peroxisomal enzyme induction by Fenofibrate is the same in the wild type and transgenic mice.
3   However, in Figure 4 of the paper the mice treated with WY-14,643 instead of Fenofibrate were
4   presented for the peroxisome proliferator marker enzyme PMP70 in total liver protein gel. There
5   appear to be more PMP70 in the transgenic mice than wild type mice as a baseline. The PMP70
6   appeared to be similar after WY-14,643 treatment. However, only one gel was given and no
7   other quantitation was given by the authors.
8
9   The authors state that "in addition WY-14,643 and Fenofibrate treatment produced
10   similar effect to the liver specific humanized PPARα mouse line (Cheung et al 2004)."
11   However, the results were not the same between Fenofibrate and WY-14,643 and the mouse line
12   used by Cheung et al. had background differences in response and pathology. In one figure in
13   the paper there appears to be a difference in background level of serum total triglyceride between
14   the wild type and hPPARα^{PAC} mice that the authors do not note. The power of using such few
15   mice does not help to sort out any significant differences in background level of triglycerides.
16   The authors note that WY-14,643 treatment also resulted in decreased serum triglycerides levels
17   in hPPARα^{PAC} mice consistent with the induction of expression of genes encoding fatty acid
18   metabolism and that the hypolipidemic effects of fibrates are generally explained by increased
19   expression of lipoprotein lipase (LPL) and decreased expression of apolipoprotein C- III (Apo
20   C-III) (Auwerx et al., 1996). However, the alteration of these genes by WY-14,643 treatment
21   was only observed in wild type mice and not in hPPARα^{PAC} mice suggesting that the
22   hypolipidemic effect observed in hPPARα^{PAC} mice are not through LPL and APO C-III. The
23   authors do not note that there could be a difference in the regulation of these pathways by the
24   transgene rather than how the normal gene is regulated and the pathways it affects. The reason
25   for examining this question with WY-14,643 treatment rather than with Fenofibrate treatment is
26   not addressed by the authors, especially since the other "markers" of peroxisomal gene induction
27   appear to be affected by Fenofibrate in the wild type and hPPARα^{PAC} mice.
28
29   Hepatomegaly was reported to be observed in the hPPARα^{PAC} mice following two weeks
30   of WY-14,643 treatment as revealed by the increase liver to body weight ratio compared to
31   untreated hPPARα^{PAC} mice but to be markedly lower when compared to wild type mice under
32   the same treatment. Histologically, the livers of the wild type mice treated with WY-14,643
33   were hypertrophic with clear eosinophilic regions with these phenotypic effects were observed in
34   both wild-type and hPPARα^{PAC} mice. The % liver/body weight was reported to increase from ~
35   4% in wild type mice to ~ 9% after WY-14,643 treatment and from ~ 4% in hPPARα^{PAC} to little
36   less that 6% after treatment with WY-14,643. In wild type mice treated with WY-14,643 the
37   labeling index was 21.8% compared with 1.1% in untreated wild type controls. In hPPARα^{PAC}
38   mice, WY-14,643 treatment was reported to give an average labeling index of 1.0% compared
39   with 0.8% in the untreated control hPPARα^{PAC} mice. Treatment with WY-14,643 treatment was
40   reported to result in a marked induction in the expression of CDK4 and cyclin D1 in the livers of
41   wild-type mice but to be unaffected hPPARα^{PAC} mice treated with WY-14,643. These data
42   were reported to be in agreement with the liver-specific PPARα-humanized mice that showed not
43   increase in incorporation of BrdU into hepatocytes upon treatment with WY-14,643 (Cheung et

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1   al. 2004) and further confirmed that activation of hPPARα dose not induce hepatocyte
2   proliferation. However, the authors present a figure an example with one liver each with no
3   quantitation given by the authors for BrdU incorporation. It is not clear whether the pictures
4   were taken from the same area of the liver or how representative they are. The numbers of mice
5   were never reported for the labeling index.   The data presented do suggest that there was
6   hypertrophy and hepatomegaly in the humanized mice and but not proliferation in this particular
7   WY,-14643 model. Of interest would be investigation of proliferation by other peroxisome
8   proliferators besides WY-14,643 at this necrogenic dose as it is WY that is the anomaly to
9   continue with proliferation at 2 weeks. The photomicrographs presented by the authors are so
10 small and at such low magnification that little detail can be discerned from them. There are no
11 portal triads or central veins to orient the reader as to what region of the liver has been affected
12 and where if any there would be hepatocellular vacuolization
13
14   To determine whether peroxisome proliferation occurred in the hPPARα^{PAC} mice upon
15 administration of PPs, Yang et al. (2007b) examined by Western Blot analysis the protein levels
16 of the major peroxisomal membrane protein 70 (PMP70) a marker of peroxisome proliferation).
17 After two weeks treatment of 1000 ppm WY-14,643, induction of PMP70 was reported to be
18 observed in the wild type mice as well as in hPPARα^{PAC} mice. The authors suggested that this
19 result indicates that peroxisomal proliferator treatment induced peroxisomal proliferation in
20 hPPARα^{PAC} mice. The results of this study indicate that hepatomegaly and peroxisome
21 proliferation occur in this humanized mouse model when treated with large concentrations of
22 WY-14,643. Thus these results are inconsistent with claims that peroxisome proliferators cannot
23 cause hepatomegaly or peroxisome proliferation in humans or that humans are refractory to these
24 effects. Like the lipid effects, they show a broader spectrum of effects may occur in humans and
25 decreases the specificity of these effects as species specific. However due to the model
26 compound being WY-14,643 at a necrogenic dose of 1000 ppm, the effect may not be seen in
27 humans using the lower potency peroxisome proliferators. It would have been useful for this
28 study to include and examination of these effects with Fenofibrate rather than WY-14,643 and
29 then attempting to extrapolate such effects to other peroxisome proliferators. The authors often
30 attribute the effects of peroxisome proliferators to those reactions induced by WY-14,643 and do
31 not acknowledge that the changes induced by WY-14,643 may be different –especially in regards
32 to hepatocellular DNA synthesis in which other peroxisome proliferators can cause liver tumors
33 without sustained proliferation that WY-14,643 induces, especially at a necrogenic dose
34
35   Yang et al. (2007b) report the results of induction of various genes by WY-14.643 in wild
36 type and hPPARα^{PAC} mice by microarray analysis followed by confirmation and quantitation by
37 qPCR and report that more genes were induced by WY-14,643 in wild type mice than in
38 hPPARα^{PAC} mice. They report that "importantly, the oncogene c-myc was not induced in
39 hPPARα^{PAC} mice. Moreover, genes encoding cell surface proteins such as Anxa2, CD39, CD63,
40 Ly6D, and CD24a, and several other genes such as *Cidea, Cidec, Dhrs8 and Hsd11b* were also
41 not induced in hPPARα^{PAC} mice. Interestingly, Sult2a1 was only induced in hPPARα^{PAC} mice
42 and not in WT mice; this gene is also induced in human hepatocytes by PP (Fang et al., 2005).
43 The regulation of several of these genes has previously been demonstrated through a PPARα-

1   dependent mechanism. Additional studies will be necessary to fully explore the molecular
2   regulatory mechanism and the functional implication associated with these differently regulated
3   genes." As stated in Section 3.2.2 above, there are several limitations for interpretations of the
4   results such as those presented by Yang et al. (2007b) which include the lack of phenotypic
5   anchoring for the results. The authors have shown changes from whole liver and have listed
6   changes in genes between wild type and humanized mice on a PPAR -/- background that in itself
7   with bring about changes in gene expression. The authors acknowledge difficulties in
8   determining what their reported gene changes mean. The authors do not indicated the context of
9   how the mice were treated, whether these are pooled results, and when the samples were taken.
10   It is assumed to be whole liver.
11
12   Yang et al. (2007b) report that "activation of PPARα alters hepatic miRNA expression
13   (Shah et al., 2007). They report that let-7C, a miRNA critical in cell growth and shown to target
14   c-myc, was inhibited by WY-14,643 treatment in wild type mice and that the expression levels of
15   both pri-let-7C and mature let-7C were significantly higher in hPPARα^{PAC} mice compared to
16   wild type mice. Treatment with WY-14,643 was reported to decrease the expression of Pi-let-7C
17   and mature let-7C in wild type mice but in hPPARα^{PAC} mice. The authors note that "in addition,
18   the induction of *c-myc* by WY-14,643 treatment in wild type mice did not occur in WY-14,643
19   treated hPPARα^{PAC} mice. This is in agreement with the previous observation in liver-specific
20   humanized PPARα (Shah et al 2007) and further indicates the activation of human PPARα does
21   not cause a change in hepatic miRNA and c-*myc* gene expression." A qPCR analysis of pri-let-
22   7C following 2 weeks WY-14,632 treatment was reported for wild type and hPPARα^{PAC} mice
23   (n=3-4). There appeared to be ~ 20 times more Let-7C expression in hPPARα^{PAC} mice than
24   control wild mice as a baseline.   The gel given by the authors showed a very small difference in
25   wild type mice in Let-7C northern blot analysis between a control wild type and WY-14,643-
26   treated wild type mouse.   There appeared to be no difference in the hPPARα^{PAC} mice between
27   control and WY-14,643 treatment and a larger stained area than the control wild type mice. The
28   relative c-my expression between the hPPARα^{PAC} mice and wild type control mice did not
29   correlate with changes in Let-7C expression. Thus, the amount of decrease by treatment with
30   WY-14,632 in wild type mice appeared to be extremely small compared to the much greater
31   baseline expression in the hPPARα^{PAC} mice. The change brought by WY-14,632 treatment in
32   wild type mice was a small change compared to the 20-fold greater baseline expression in the
33   hPPARα^{PAC} mice. The authors stated that the expression of the c-Myc regulator was higher in the
34   hPPARα^{PAC} mice indicating over regulation of cell division and an inability for hepatocytes to
35   proliferate. However, their results showed that there was a greater difference in regulatory
36   baseline function of the PPAR using this paradigm and this construct. Are these differences due
37   to human PPAR or to the way PPAR was put back into PPAR-/- mouse and expected to
38   function? If the experiment included mouse PPAR put back in this way on a null background,
39   what would such an experiment show? Are these results representative of the PPAR or how it is
40   now controlled and expressed? In addition, what would the study of other peroxisome
41   proliferators besides WY-14,643 show in regard to changes in miRNA. Are these results
42   reflective of a just the transient effect that is prolonged in a special case?   As discussed in
43   Section 3.2.2. there are issues with microarray data in addition to the newly emerging field of

1 miRNA arrays which include phenotypic anchoring and whether they are from whole liver or
2 pooled samples. The results given in this report are for relative Let-7C expression given and not
3 absolute values. The changes in baseline Let-7C expression between the wild type and the
4 hPPARα^{PAC} mice did not correlate with the magnitude of difference in northern blot analysis and
5 did not correlate at all with c-myc expression reported in this study. Thus, a direct correlation
6 between the effect of Let-7C expression and function and effects from WY-14,643 was not
7 supported. The relative expression was reported but the variation of baseline expression of the
8 "PPAR controlled genes" was not. Given that one of the first figures reported a large difference
9 between animals in expression of the human PPAR gene in the transgenic animals, how did this
10 difference affect the results given here as relative changes downstream?
11
12   Yang et al. (2007b) conclude that the hPPARα^{PAC} mice represent the most relevant model
13 for humans since, the tissue distribution of PPARα is similar to that observed in wild-type mice
14 and the hPPARα in hPPARα^{PAC} mice is under regulation of its native promoter. Indeed up-
15 regulation of hepatic mPPARα in wild type mice by fasting was mirrored by the hPPARα in
16 hPPARα^{PAC} mice. However, there was no demonstration that the artificial chromosome that is
17 replicating along with other DNA is controlled sterically by the same control since it is not on
18 the mouse genome in the same place as the native PPAR. There is also not a demonstration of
19 how stable the baseline of PPAR DNA expression is in this mouse model – does it vary as much
20 or more than native PPAR between mice? The authors state that "induction of PPARα target
21 genes for fatty acid metabolism and a decrease in serum triglycerides by PP in hPPARα^{PAC} mice
22 indicates that hPPARα is functional in the mouse environment with respects to regulation of fatty
23 acid metabolism. This is in agreement with the liver-specific PPARα humanized mice that also
24 exhibit these responses (Cheung et al., 2004). Indeed the DNA binding domain of hPPARα is
25 100% homologous with that of the mouse suggesting that both bind to the same PPRE binding
26 site in the promoter region of target genes. Transfection of hPPAR into murine hepatocytes
27 increased PPs induced peroxisome proliferation related effects (Macdonald et al., 1999). These
28 results suggest that hPPARα and mPPARα do not differ in induction of target genes with known
29 PPRE." However, replacement with human PPAR in the Cheung et al. model is not sufficient to
30 prevent the same types of toxicity as seen with PPAR knockouts on the hepatocytes such as
31 steatosis.
32
33   Yang et al. (2007b) note that "the increased LPL an decreased expression of apo C-III are
34 proposed to explain the hypolipidemic effects of PPS (Auwerx et al., 1996). However,
35 hPPARα^{PAC} mice treated with PP exhibit lowered serum triglycerides without alteration of the
36 expression of LPL and apoC-III. This indicates the hypolipidemic effects in rodents are
37 mediated via other molecular regulatory mechanisms. It is also suggested that the activation of
38 PPARα by PPs stimulates hepatic fatty acid oxidation and thereby diminishing their
39 incorporation into triglycerides and secretion of VLDL (Froyland et al., 1997). Consistent with
40 this idea, a robust induction of the genes encoding enzymes for fatty acid oxidation by PP in
41 hPPARα^{PAC} mice were observed. Thus, the exact mechanism by which PPs exert their
42 hypolipidemic effects needs reexamination." However, the use of two different peroxisome
43 proliferators (i.e., WY-14,643 and Fenofibrate) for two types of effects (peroxisomal and lipid)

1   may be the cause of some paradoxes here in terms of MOA for lipid effects. The baseline
2   differences in the hPPARα[PAC] mice for serum total triglicerides was not explored by these
3   authors and the small number of animals used make conclusions difficult about the magnitude of
4   difference. The differences in baseline expression for LPL are not discernable in the graphic
5   representation of the results.
6
7   Yang et al. (2007b) note that "on the other hand, the difference in the affinity of ligands
8   for the human and mouse PPARα receptor was proposed to account for the species difference.
9   The ligand binding domain of hPPARα is 94% homologous with that of the mouse. In vitro
10 transactivation assays have previously shown that WY has a higher affinity for rodent PPARα
11 than human PPARα, while Fenofibrate has similar affinity for rodent and human PPARα
12 (Shearer and Hoekstra 2003; Sher et al., 1993). In the present study WY and Fenofibrate exhibit
13 the same capacity to induce known PPARα target genes in the liver, kidney and heart in both
14 wild-type and hPPARα[PAC] mice."   The statement by the authors that Fenofibrate and WY-
15 14,643 had the same affinity "as shown by this study" is not true. The two treatments were not
16 studied for the same enzymes or genes in the data reported in the study. Both WY-14,643 and
17 Fenofibrate can induce PPARα targets but it was not shown to the same extent. Yang et al.
18 (2007b) state that "This is in agreement with the liver-specific PPARα humanized mice that also
19 exhibit a similar capacity to induce PPARα target genes in liver by WY and Fenofibrate (Cheung
20 et al., 2004). Thus, the ligand affinity difference between mouse and human PPARα may not be
21 critical under the conditions of these studies." Alternatively, these results could reflect that these
22 studies were conducted with two different agonists with different affinities and responses due to
23 receptor activation.
24
25   Finally, a useful comparison to make are the differences between wild type mice, PPARα
26 -/- mice that serve as the background for the transgenic human mouse models, and both
27 transgenic models. The small and variable number of animals examined in these studies is
28 readily apparent. The results of the Cheung et al. (2004) humanized mouse model and those
29 reported for Yang et al. (2007b) show differences in the study designs including PPARα
30 agonists studied for particular effects and results reported for similar treatments.

Comparison between results for Yang et al. 2007b and Cheung et al. 2004:**

Effect	Wild type mice	PPAR -/- knockout mice	Humanized mice (liver only)	Humanized PAC mice
Triglycerides	**Cheung (n=6-9)** Control 145 mg/ml 0.1% WY-14,643 60mg/ml (2 wks) 0.2% Fenofibrate 85 mg/ml (2 wks) **Yang (n=4-6)** Control 95 mg/ml 0.1 % WY-14,643 55mg/ml (2wks)	**Cheung (n=6-9)** Control 100 mg/ml 0.1% WY-14,643 115 mg/ml (2 wks) 0.2% Fenofibrate 85 mg/ml (2 wks)	**Cheung (n=6-9)** Control 175 mg/ml 0.1%WY-14,643 60 mg/ml (2 wks) 0.2% Fenofibrate 85 mg/ml (2 wks)	**Yang (n=4-6)** Control 120 mg/ml 0.1%WY-14,643 75 mg/ml (2 wks)
BrdU incorporation	**Cheung (n=5)** Control 1.6 % 0.1% WY-14,643 57.9 % (8 wks) **Yang (n=4-6)** Control 1.1 % 0.1% WY-14,643 21.8 % (2 wks)	Not done	**Cheung (n=5)** Control 1.6 % 0.1% WY-14,643 2.8 % (8wks)	**Yang (n=4-6)** Control 0.8 % 0.1% WY-14,643 1.0% (2 wks)
Hepatomegaly* (% liver body weight ratio)	**Cheung (n=5-9)** Control 4 % 0.1% WY-14,643 11 % (2 wks) 0.2% Fenofibrate 8.5% (2 wks)	**Cheung (n=5-9)** Control 5 % 0.1% WY-14,643 5 % (2 wks) 0.2% Fenofibrate 5.5% (2 wks)	**Cheung (n=5-9)** Control 4.5 % 0.1% WY-14,643 7 % (2 wks) 0.2% Fenofibrate 7 % (2 wks)	

	Yang **(n=4-6)** Control 4 % 0.1% WY-14,643 9 % (2 wks)			**Yang** **(n=4-6)** Control 4 % 0.1% WY 6 % (2 wks)

*Percentages are approximate values extrapolated from figures for hepatomegaly.** The ages of the humanized knockout mice are not given for Cheung et al. 2004 but are 8-10 weeks for Yang et al. 2007b.

1   As shown above, the effect on the PPARα-/- by the knockout included decreased
2   triglyceride levels and slightly increased liver weight. Although treatment with WY-14,643 and
3   Fenofibrate were reported to decrease triglyceride levels in wild-type mice, paradoxically so did
4   knocking out the receptor. Exposures to WY-14,643 appeared to induce a slight increase and
5   Fenofibrate a slight decrease in triglyceride levels in PPARα -/- mice but the variability of
6   response and small number of animals in the experiments limited that ability to discern a
7   quantitative difference in the treatments. In the study by Cheung et al. (2004) it appears that the
8   insertion of humanized PPARα restored the baseline and treatment responses for triglyceride
9   levels. Overall, the results reported by Yang et al. (2007b) appeared to have a lower level of
10 triglycerides in control wild type mice that was similar in magnitude to the treatment effect
11 reported by Fenofibrate by Cheung et al. (2004). However, there also appeared to be restoration
12 of this effect in the humanized mouse model of Yang et al. (2007b). In regard to DNA synthesis,
13 both Cheung et al. (2004) and Yang et al. (2007b) only gave results for WY-14,643 and for
14 different durations of exposure so they were not comparable. It appeared that ~ 60 % of
15 hepatocytes were labeled by 8 weeks of WY-14,643 treatment (Cheung et al., 2004) compared to
16 ~ 20% after 2 weeks of exposure. Again this highlights the difference between using WY-14,643
17 as a model for the PPARα as a class at times when almost all other PPARα agonists have ceased
18 to increase DNA synthesis or have reductions in this parameter. The background changes due to
19 the PPARα -/- knockout were not reported so that the effects of the knockout could not be
20 ascertained. It appeared that insertion of humanized PPARα did not results in restoration of WY-
21 14,643 –induced DNA synthesis. The correlation with this parameter and any focal areas of
22 necrosis were not discussed by the authors of the study. In regard to hepatomegaly, Fenofibrate
23 and WY-14,643 appeared to both give an increase in liver weight in the humanized mouse model
24 of Cheung et al., (2004) with little effect in the knockout mouse. For Fenofibrate there was little
25 difference in liver weight gain in the wild type mouse and that of the humanized mouse model of
26 Cheung et al., (2004).   However, Fenofibrate was not tested in the humanized mouse model of
27 Yang et al. (2007b). In that model only WY-14,643 was used but there was still an increase in
28 liver weight. Thus, in terms of effects on liver weight gain and triglyceride levels both models
29 gave comparable results and appeared to indicate that insertion humanized PPARα would restore
30 some of the effects of the knockout. However, the results from both experiments highlight the
31 need for adequate numbers of animals and other PPARα agonists to be tested besides WY-14,463
32 at such a high dose and certainly for longer periods of time to ascertain whether such
33 manipulations will affects carcinogenicity.
34
35 *3.4.1.4 NF-κB activation*
36
37 NF-κB activation has also been proposed as a key event in the induction of liver cancer
38 through PPARα activation. As discussed in Sections 3.2.6. and 3.4.3.3., activation of the NF-κB
39 pathway is implicated in carcinogenesis, non-specific for a particular MOA for liver cancer, and is
40 context dependent on its effects. Its specific actions depend on the cell type and type of agent or
41 signal that activates translocation of the complex. NF-κB is not only involved in biological
42 processes other than tumor induction, but also exhibits some apparently contradictory behaviors
43 (Perkins and Gilmore, 2006). Although many studies point to a tumor-promoting function of NF-

1 κB subunits, evidence also exists for tumor suppressor functions. NF-κB actions are associated
2 with TNF and JNK among many other cell signaling systems and molecules and it has functions
3 that alter proliferation and apoptosis. NF-κB activation reported in some studies may be
4 associated with early Kupffer cell responses and be associative but not key events in the
5 carcinogenic process. However, most assays look at total NF-κB expression in the whole liver
6 and at the early periods of proliferation and apoptosis. The origin of the NF-κB is crucial as to its
7 effect in the liver. For instance hepatocyte specific deletion of IKKβ increased DEN-induced
8 hepatocarcinogenesis but a deletion of IKKβ in both hepatocytes and Kupffer cells however, had
9 the opposite effect (Maeda et al., 2005).
10
11 *3.4.1.5. Phenotype as an indicator of a PPARα MOA*
12
13   As discussed previously (see Sections 3.1.5, and 3.1.8.) foci of altered hepatocytes (FAH)
14 precede both hepatocellular adenomas and carcinomas in rodents and, in humans with chronic
15 liver diseases that predispose them to hepatocellular carcinomas. Striking similarities in specific
16 changes of the cellular phenotype of preneoplastic FAH are emerging in experimental and human
17 hepatocarcinogenesis, irrespective of whether this was elicited by chemicals, hormones,
18 radiation, viruses, or, in animal models, by transgenic oncogenes or *Helicobacter hepaticus*.
19 Several authors have noted that the detection of phenotypically similar FAH in various animal
20 models and in humans prone to developing or bearing hepatocellular carcinomas favors the
21 extrapolation from data obtained in animals to humans (Bannasch et al., 2003; Su and Bannasch,
22 2003; Bannasch et al., 2001). In regard to phenotype by tincture Caldwell and Keshava (2006)
23 state:
24
25   In addition, the term "basophilic" in describing preneoplastic foci or tumors
26   can be misleading. The different types of FAH have been related to three
27   main preneoplastic hepatocellular lineages: 1) the glycogenotic-basophilic
28   cell lineage, 2) its xenomorphic-tigroid cell variant, and 3) the amphophilic-
29   basophilic cell lineage. Specific changes of the cellular phenotype of the
30   first two lineages of FAHs are similar in experimental and human
31   hepatocarcinogenesis, irrespective of whether they were elicited by DNA-
32   reactive chemicals, hormones, radiation, viruses, transgenic oncogenes and
33   local hyperinsulinism as described by the first two FAH's and this
34   similarity favors extrapolation from data obtained in animals to humans
35   (Bannasch et al., 2003; Su and Bannasch, 2003; Bannasch et al., 2001). In
36   contrast, the amphophilic cell lineage of hepatocarcinogenesis has been
37   observed mainly after exposure of rodents to peroxisome proliferators or to
38   hepadnaviridae (Bannasch et al., 2001).
39
40   Bannasch (1996) describes "amphophilic" FAH and tumors induced by
41   peroxisome proliferators to maintain the phenotype as the foci progress to
42   tumors. They are glycogen poor from the start with increased numbers of
43   mitochondria, peroxisomes and ribosomes. The author further states that

1 the "homogenous basophilic" descriptions by others of foci induced by WY
2 are really amphophilic. Agents other than peroxisome proliferators can
3 induce "acidophilic" or "eosinophilic" (due to increased smooth
4 endoplasmic reticulum) or glycognotic foci which tend to progress to
5 basophilic stages (due to increased ribosomes).

7 Tumors and foci induced by peroxisome proliferators have been suggested
8 to have a phenotype of increased mitochondrial proliferation and
9 mitochondrial enzymes (thyromimetic rather than insulinomimetic)
10 (Keshava and Caldwell, 2006).

12 Tumors from peroxisome proliferators in Kraupp-Grasl et al. (1990) and Grasl-Kraupp et
13 al. (1993) for rat liver tumors were characterized as weakly basophilic with some eosinophilia
14 and as similar to the description given by Bannasch et al as amphophilic. However, a number of
15 recent studies indicate that other "classic" peroxisome proliferators may have a different
16 phenotype than has been attributed to the class through studies of WY-14,643. A recent study of
17 di (2-ethylhexyl) phthalate (DEHP), another peroxisome proliferator assumed to induce liver
18 tumors through activation of the PPARα receptor, reported the majority of liver FAH to be of the
19 first two types after a lifetime of exposure to DEHP with a dose-related tendency for increased
20 numbers of amphophilic FAHs in rats (Voss et al., 2005). As stated previously, the MOA of
21 DEHP-induced liver tumors in mice also appears to not be dependent on PPARα activation.

23 Michel et al. (2007) report the phenotype of tumors and foci in rats treated with clofibric
24 acid at a very large dose (5000 ppm for 20 months) and note that in controls the first type of foci
25 to appear was tigroid on day 264 and their incidence increased with time representing the most
26 abundant type in this group. They report no adenomas or carcinomas after up to 607 days after
27 given saline injection in the control animals. DEN treatment was examined up to 377 days only
28 with tigroid, eosinophilic and clear cell foci at that time observed at that time. Clofibric acid was
29 examined up to 607 days and tigroid and clear cell foci were reported to be the first to appear on
30 day 264 with no other foci class. By day 377 there were tigroid, eosinophilic and clear cell foci
31 but no basophilic foci reported with clofibric acid treatment and, although only a few animals
32 were examined, 2/5 had adenomas but not carcinomas. By day 524 all types of foci were seen
33 (including basophilic for the first time) and there were adenomas and carcinomas in 2/5 animals.
34 By 607 days a similar pattern was observed without adenomas but 3/6 animals showing
35 carcinomas. Although the number of animals examined is very small, these results indicate that
36 clofibric acid was not inducing primarily "basophilic foci" as reported for peroxisome
37 proliferators but the first foci are tigroid and clear cell foci. Basophilic foci did not appear till
38 day 524 similar to control values for foci development and distribution. However unlike
39 controls, clofibric acid induced eosinophilic and clear cell foci earlier. This is inconsistent with
40 the phenotype ascribed to peroxisome proliferators as exemplified by WY-14,643.

42 In regard to γ-glutathione –S-transferase (GST-π) and γ-transpeptidase (GGT), Rao et al
43 (1986) fed 2 male F344 rats a diet of 0.1% WY-14,643 for 19 months or 3 F344 rats 0.025%

1 Ciprofibrate for 15-19 months and reported "altered areas,"(AA) "neoplastic nodules" (NN), and
2 hepatocellular carcinomas (HCC). For WY-14,643 treatment 107 AA, 75 NN, and 5 HCC, and
3 for Ciprofibrate treatment 107 AA, 27 NN, and 16 HCC were identified. In the WY-14.643-
4 treated rats, HCC, and NN were both GGT and GST-π negative (96-100%) with 87% of AA was
5 negative for both. In Ciprofibrate-treated rats NN and HCC were negative for both markers
6 (95%) but only 46% of AA were negative for both markers. Thus, a different pattern for tumor
7 phenotype was reported for WY-14,643 and another peroxisome proliferator, Ciprofibrate, in this
8 study as well.
9
10   In addition, GGT phenotype is reported to not be specific to weakly basophilic foci. GGT
11 staining was reported to be negative in eosinophilic tumors after initiation and promotion.
12 Kraupp-Grasl et al. (1990) note differences among PPARα agonists in their ability to promote
13 tumors and suggest they not necessarily be considered a uniform group. Caldwell and Keshava
14 (2006) suggest that the reports of a simple designation of "basophilic" is not enough to associate
15 a foci as caused by peroxisome proliferators [Bannasch, 1996; Grasl-Kraupp et al.,1993; Kraupp-
16 Grasl et al., 1990]. Increased basophilia of tumors and increased numbers of carcinomas is
17 consistent with the progressive basophilia described by Bannasch (1996), as many adenomas
18 progress to carcinomas."
19
20   It should be noted that the amphophilic foci and tumors described by Bannasch et al. were
21 primarily studied in rats. Morimura et al. (2006) noted that WY-14,643 induced diffusely
22 basophilic tumors in mice and therefore identified the WY-14,643 tumors in a way consistent
23 with the descriptions of amphophilic tumors by Bannasch et al.   The tumor induced by WY-
24 14,643 in their humanized mouse was reported to be similar to those arising spontaneously in the
25 mouse.   However, the mouse response could differ from the rat, especially for PPARα agonists
26 other than WY-14,643.
27
28   H-ras activation and mutation studies have attempted to assign a pattern to peroxisome
29 proliferator-induced tumors as noted in Section 2.3.3.2., above. However also as noted in
30 Section 2.3.3.2, the genetic background of the mice used, the dose of carcinogen and the stage of
31 progression of "lesions" (i.e., foci vs. adenomas vs. carcinomas) may affect the number of
32 activated H-ras containing tumors which develop. Fox et al. (1990) note that tumors induced by
33 Ciprofibrate (0.0125% diet, 2 years) had a much lower frequency of H-ras gene activation than
34 those that arose spontaneously (2-year bioassays of control animals) or induced with the
35 "genotoxic" carcinogen benzidine-2 HCl (120 ppm, drinking H_2O, 1 yr) and that the
36 Ciprofibrate-induced tumors were reported to be more eosinophilic as were the surrounding
37 normal hepatocytes than spontaneously occurring tumors. Anna et al. (1994) also stated that
38 mice treated with Ciprofibrate had a markedly lower frequency of tumors with activated H-ras
39 but that the spectrum of mutations in tumors was similar those in "spontaneous tumors."   Hegi et
40 al. (1993) tested Ciprofibrate-induced tumors from Fox et al. (1990) in the NIH3T3
41 cotransfection-nude mouse tumorigenicity assay and concluded that ras protooncogene activation
42 were not frequent events in Ciprofibrate-induced tumors and that spontaneous tumors were not
43 promoted with it. Stanley et al. (1994) studied the effect of Methylclofenapate (MCP), a

1   peroxisome proliferator, in B6C3F1 (relatively sensitive) and C57BL/10J (relatively resistant)
2   mice for H-ras codon 61 point mutations in MCP-induced liver tumors (hepatocellular adenomas
3   and carcinomas). In the B6C3F1 mice, ~ 24% of MCP-induced tumors had codon 61 mutations
4   and for C57BL/10J mice ~ 13%. The findings of an increased frequency of H-ras mutation in
5   carcinomas compared to adenomas in both strains of mice is suggestive that these mutations were
6   related to stage of progression. Thus in mice, the phenotype of tumors did not appear to be
7   readily distinguishable from spontaneous tumors based on tincture for peroxisome proliferators
8   other than WY-14,643, but did have more of a signature in terms of H-ras mutation and
9   activation.
10
11   The expression of c-Jun has been used to discern TCE tumors from those of its
12 metabolites. However, as pointed out by Caldwell and Keshava (2006), although Bull et al.
13 (2004) have suggested that the negative expression of c-*jun* in TCA-induced tumors may be
14 consistent with a characteristic phenotype shown in general by peroxisome proliferators as a
15 class, there is no supporting evidence of this. While increased mitochondrial proliferation and
16 mitochondrial enzymes (thyromimetic rather than insulinomimetic) properties have been ascribed
17 to peroxisome proliferator-induced tumors, and the studies cited in Bull et al. (2004) have not
18 examined TCA-induced tumors for these properties.
19
20 *3.4.1.6. Human relevance*
21
22   In its framework for making conclusions about human relevance, the U.S. EPA Cancer
23 Guidelines (U.S. EPA 2005) asks that critical similarities and differences between test animals
24 and humans be identified. Humans possess PPARα at sufficient levels to mediate the human
25 hypolipidemic response to peroxisome-proliferating fibrate drugs. Fenofibrate and Ciprofibrate
26 induce treatment related increases in liver weight, hypertrophy, numbers of peroxisomes,
27 numbers of mitochondria, and smooth endoplasmic reticulum in cynomologous monkeys at 15
28 days of exposure (Hoivik et al., 2004). Given the species difference in the ability to respond to a
29 mitogenic stimulus such as partial hepatectomy (see Section 3.3.) lack of hepatocellular DNA
30 synthesis at this time point is not unexpected and as Rusyn et al. (2006) note examination at
31 differing time point may produce differing results. It is therefore generally acknowledged that "a
32 point in the rat and mouse key events cascade where the pathway is biologically precluded in
33 humans in principle cannot be identified."(Klaunig et al. 2003; NAS 2006). Thus, from a
34 qualitative standpoint, the effects described above are plausible in humans.
35
36   As for quantitative differences, there are two key issues. First, as stated in the Cancer
37 Guidelines, when considering human relevance, "Any information suggesting quantitative
38 differences between animals and humans is flagged for consideration in the dose-response
39 assessment." Therefore, while Klaunig et al. (2003) and NAS (2006) go on to suggest that
40 "this mode of action is not likely to occur in humans based on differences in several key steps
41 when taking into consideration kinetic and dynamic factors," under the Cancer Guidelines,
42 such "kinetic and dynamic factors" need to be made explicit in the dose-response assessment,
43 and should not be part of the qualitative characterization of hazard. Second, the discussion

1 above points to the lack of evidence supporting associations between the postulated events and
2 carcinogenic potency. Thus, because inter-species differences in carcinogenicity do not appear
3 to be associated with inter-species differences in postulated events, they do not provide reliable
4 metrics with which to make inferences about relative human sensitivity.
5
6 **3.4.2. Other TCE Metabolite Effects That May Contribute to its Hepatocarcinogenicity**

7 While the focus of most studies of TCA has been its effects on peroxisomal proliferation,
8 DCA has been investigated for a variety of effects that are also observed either in early stages of
9 oncogenesis (glycogen deposition) or conditions that predispose patients to liver cancer. Some
10 studies have examined microarray profiles in attempt to study the MOA or TCE (see section 3.2.2
11 for caveats regarding such approaches). Caldwell and Keshava have provided a review of these
12 studies which is provided below.
13
14 *3.4.2.1. DCA effects and glycogen accumulation correlations with cancer*

15 As noted previously, DCA administration has been reported to increase the observable
16 amount of glycogen in mouse liver via light microscopy and, although to not be primarily
17 responsible for DCA-induced liver mass increases, to be increase whole liver glycogen as much
18 by 50% (Kato-Weinstein et al., 2001). Given that TCE and DCA tumor phenotypes indicate a role
19 for DCA in TCE hepatocarcinogenicity (see Section 2.3.3.2., above), Caldwell and Keshava
20 (2006) described the correlations with effects induced by DCA that have been associated with
21 hepatocarcinogenicity.
22
23 A number of studies suggest DCA-induced liver cancer may be linked to its
24 effects on the cytosolic enzyme glutathione (GST)-S-transferase-zeta. GST-
25 zeta is also known as maleylacetoacetate isomerase and is part of the
26 tyrosine catabolism pathway whose disruption in type 1 hereditary
27 tyrosinemia has been linked to increased liver cancer risk in humans. GST-
28 zeta metabolizes maleylacetoacetate (MAA) to fumarylacetoacetate (FAA)
29 which displays apoptogenic, mutagenic, aneugenic, and mitogenic activities
30 (Bergeron et al., 2003; Jorquera and Tanguary, 2001; Kim et al 2000).
31 Increased cancer risk has been suggested to result from FAA and MAA
32 accumulation (Tanquary et al 1996). Cornett et al. (1999) reported DCA
33 exposure in rats increased accumulation of maleylacetone (a spontaneous
34 decarboxylation product of MAA), suggesting MAA accumulation.
35 Ammini et al. (2003) report depletion of the GST-zeta to be exclusively a
36 post-transcriptional event with genetic ablation of GST-zeta causing FAA
37 and MAA accumulation in mice. Schultz et al. (2002) report that
38 elimination of DCA is controlled by liver metabolism via GST-zeta in mice,
39 and that DCA also inhibits the enzyme (and thus its own elimination) with
40 young mice being the most sensitive to this inhibition. On the other hand,
41 older mice (60 weeks) had a decreased capacity to excrete and metabolize

DCA in comparison with younger ones. The authors suggest that exogenous factors that deplete or reduce GST-zeta will decrease DCA elimination and may increase its carcinogenic potency. They also suggest that, due to suicide inactivation of GST-zeta, an assumption of linear kinetics can lead to an underestimation of the internal dose of DCA at high exposure rates. In humans, GST-zeta has been reported to be inhibited by DCA and to be polymorphic (Tzeng et al 2000; Blackburn et al., 2001, 2000). Board et al. (2001) report one variant to have significantly higher activity with DCA as a substrate than other GST zeta isoforms, which could affect DCA susceptibility.

Individuals with glycogen storage disease or with poorly controlled diabetes have excessive storage of glycogen in their livers (glycogenosis) and increased risk of liver cancer (LaVecchia et., 1994; Adami et al., 1996; Wideroff et al., 1997; Rake et al., 2002). In an animal model where hepatocytes are exposed to a local hyperinsulinemia from transplanted islets of Langerhans and the remaining tissue is hypoinsulinemic, insulin induces alterations that resemble preneoplastic foci of altered hepatocytes (FAH) and develop into hepatocellular tumors in later stages of carcinogenesis (Evert et al., 2003). A number of studies have reported suppression of apoptosis, decreases in insulin, and glycogenosis in mice liver by DCA at levels that also induce liver tumors (Bull, 2004; Bull et al., 2004; Lingohr et al., 2001). In isolated murine hepatocytes, Lingohr et al. (2002) reported DCA-induced glycogenosis was dose related, occurred at very low doses ($10 \mu M$), occurred without the presence of insulin, was not affected by insulin addition, was dependent on phosphatidylinositol 3-kinase (P13K) activity, and was not a result of decreased glycogen breakdown. The authors noted that PI3K is also known to regulate cell proliferation and apoptosis in hepatocytes, and that understanding these mechanisms may be important to understanding DCA-induced carcinogenesis. They also report insulin receptor (IR) protein levels decreased to 30% of controls in mice liver after up to 52 weeks of DCA treatment. Activation of the IR is also the principal pathway by which insulin stimulates glycogen synthetase (the rate limiting enzyme of glycogen biosynthesis). However, in DCA-induced liver tumors IR protein was elevated as well as mitogen-activated protein kinase (a downstream target protein of the IR) phosphorylation. DCA-induced tumors were glycogen poor (Lingohr et al., 2001). The authors suggest that normal hepatocytes down-regulate insulin-signaling proteins in response to the accumulation of liver glycogen caused by DCA and that the initiated cell population, which does not accumulate glycogen and is promoted by DCA treatment, responds differently from normal hepatocytes to the insulin-like effects of DCA.

Gene expression studies of DCA show a number of genes identified with cell growth, tissue remodeling, apoptosis, cancer progression, and xenobiotic metabolism to be altered in mice liver at high doses (2 g/l DCA) in drinking water (Thai et al., 2001, 2003). After 4 weeks, RNA expression was altered in 4 known genes (alpha-1 protease inhibitor, cytochrome B5, stearoyl-CoA desaturase and caboxylesterase) in two mice (Thai et al., 2001). Except for Co-A desaturase, a similar pattern of gene change was reported in DCA-induced tumors (10 tumors from 10 different mice) after 93 weeks. Using cDNA microarray in the same mice, Thai et al. (2003) identified 24 genes with altered expression, of which 15 were confirmed by Northern blot analysis after 4 weeks of exposure. Of the 15 genes, 14 revealed expression suppressed two- to fivefold and included: MHR 23A, cytochrome P450 (CYP), 2C29, CYP 3A11, serum paraoxonase/arylesterase 1, liver carboxylesterase, alpha-1 antitrypsin, ER p72, GST-pi 1, angiogenin, vitronectin precursor, cathepsin D, plasminogen precursor (contains angiostatin), prothrombin precursor and integrin alpha 3 precursor. An additional gene, CYP 2A4/5, had a twofold elevation in expression. After 93 weeks of treatment with 3.5 g/l DCA, Northern blot analyses of total RNA isolated from DCA-induced hepatocellular carcinomas showed similar alteration of expression (11 of 15). It was noted that peroxisome proliferator-activated receptor (PPAR)α and IR gene expression were not changed by DCA treatment. Genes involved in glycogen or lipid metabolism were not tested.

Although it has not been possible to directly determine whether DCA is produced from TCE at carcinogenic levels, there is indirect evidence that DCA is formed from TCE *in vivo* and contributes to liver tumor development. Pretreatment with either DCA or TCE inhibits GST-zeta while TCA pretreatment does not (Schultz et al., 2002; Bull et al., 2004). TCE treatment decreased V_{max} for DCA metabolism to 49% of control levels with a 1 g/kg TCE dose resembling effects those of 0.05 g/l DCA (Schultz et al., 2002).

3.4.2.2. Genetic profiling data for TCE: gene expression and methylation status studies

Caldwell and Keshava (2006) and Keshava and Caldwell (2006) report on both genetic expression studies and studies of changes in methylation status induced by TCE and its metabolites (See sections 2.3.2. and 2.3.3, above) as well as differences and difficulties in the patterns of gene expression between differing PPARα agonists. In Section 4.2.2. (below), the effects of co-exposures of DCA, TCA and Chloroform on methylation status are discussed. In particular are concerns for the interpretation of studies which employ pooling of data as well as interpretation of "snapshots in time of multiple gene changes." For the Laughter et al. (2004) study in particular, it is not clear whether transcription arrays were performed on pooled data (no

data on variability between individual animals was provided and the methodology section of the report is not transparently written in this regard). The issue of phenotypic anchoring also arises as data on % liver/body weight indicates significant variability within TCE treatment groups, especially in PPARα-null mice.   For studies of gene expression using microarrays Bartosiewicz et al (2001) used a screening analysis of 148 genes for xenobiotic-metabolizing enzymes, DNA repair enzymes, heat shock proteins, cytokines, and housekeeping gene expression patterns in the liver in response TCE. The TCE-induced gene induction was reported to be highly selective; only Hsp 25 and 86 and Cyp2a were up-regulated at the highest dose tested. Collier et al. (2003) reported differentially expressed mRNA transcripts in embryonic hearts from Sprague-Dawley rats exposed to TCE with sequences down-regulated with TCE exposure appearing to be those associated with cellular housekeeping, cell adhesion, and developmental processes. TCE was reported to induce up-regulated expression of numerous stress-response and homeostatic genes.

For the Laughter et al. (2004) study, transcription profiles using macroarrays containing approximately 1,200 genes were reported in response to TCE exposure. Forty three genes were reported to be significantly altered in the TCE-treated wild-type mice and 67 genes significantly altered in the TCE-treated PPARα knockout mice. Out of the 43 genes expressed in wild-type mice upon TCE exposure, 40 genes were reported by the authors to be dependent on PPARα and included genes for CYP4a12, epidermal growth factor receptor, and additional genes involved in cell growth. However, the interpretation of this information is difficult because in general, PPARα knockout mice have been reported to be more sensitive to a number of hepatotoxins partly because of defects in the ability to effectively repair tissue damage in the liver (Shankar et al., 2003; Mehendale, 2000) and because a comparison of gene expression profiles between controls (wild-type and PPARα knockout) were not reported.

As stated previously, knockout mice in this study also responded to TCE exposure with increased liver weight, had increased background liver weights, also had higher baseline levels of hepatocyte proliferation than wild-type mice. Nakajima et al. (2000) reported that the number of peroxisomes in hepatocytes increased by 2 fold in wild-type mice but not in PPARα knockout mice. However, TCE induced increased liver weight in both male and female wild type and knockout mice, suggesting hepatic effects independent of PPARα activation. In regards to toxicity, after three weeks of TCE treatment (0 to1,500 mg/kg via gavage), Laughter et al. (2004) reported toxicity at the1500 mg/kg level in the knockout mice that was not observed in the wild-type mice — all knockout mice were moribund and had to be removed from the study. Differences in experimental protocol made comparisons between TCE effects and those of its metabolites difficult in this study (see Section 2.1.13 above).

As reported by Voss et al. (2006), dose-, time course-, species-, and strain-related differences should be considered in interpreting gene array data. The comparison of differing PPARα agonists presented in Keshava and Caldwell (2006) illustrate the pleiotropic and varying liver responses of the PPARα receptor to various agonists, but did imply that these responses were responsible for carcinogenesis.

1   As discussed above in Section 3.3.5 and in Caldwell and Keshava (2006), "Aberrant DNA
2   methylation has emerged in recent years as a common hallmark of all types of cancers, with
3   hypermethylation of the promoter region of specific tumor suppressor genes and DNA repair
4   genes leading to their silencing (an effect similar to their mutation) and genomic hypomethylation
5   (Ballestar and Esteller 2002; Berger and Daxenbichler 2002; Herman et al. 1998; Pereira et al.
6   2004; Rhee et al. 2002). Whether DNA methylation is a consequence or cause of cancer is a long-
7   standing issue (Ballestar and Esteller 2002). Fraga et al. (2004, 2005) reported global loss of
8   monoacetylation and trimethylation of histone H4 as a common hallmark of human tumor cells;
9   they suggested, however, that genomewide loss of 5-methylcytosine (associated with the
10   acquisition of a transformed phenotype) exists not as a static predefined value throughout the
11   process of carcinogenesis but rather as a dynamic parameter (i.e., decreases are seen early and
12   become more marked in later stages)."
13
14   Although little is known about how it occurs, a hypothesis has also been proposed that that
15   the toxicity of TCE and its metabolites may arise from its effects on DNA methylation status. In
16   regard to methylation studies, many are co-exposure studies as they have been conducted in
17   initiated animals, and as stated above, some are very limited in regard to the reporting and conduct
18   of the study. Caldwell and Keshava (2006) review the body of work regarding TCE, DCA and
19   TCA. Methionine status has been noted to affect the emergence of liver tumors. As noted by
20   Counts et al. (1996) a choline/methionine deficient diet for 12 months did not increase liver tumor
21   formation in C3H/HeN mice but is tumorigenic to B6C3F1 mice. Tao et al (2000) and Pereira et
22   al (2004) have studied the effects of excess methionine in the diet to see if it has the opposite
23   effects as a deficiency (i.e. and reduction in a carcinogenic response rather than enhancement). As
24   noted above for Tao et al. (2000), the administration of excess methionine in the diet is not
25   without effect. The data of Tao et al. (2000) suggest that % liver/body weight ratios are affected
26   by short-term methionine exposure (300 mg/kg) in female B6C3F1 mice. Pereira et al (2004)
27   reported that very high level of methionine supplementation to an AIN-760A diet, affected the
28   number of foci and adenomas after 44 weeks of co-exposure to 3.2.g/l DCA. While the highest
29   concentration of methionine (8.0 g/kg) was reported to decrease both the number of DCA-induce
30   foci and adenomas, the lower level of methionine co-exposure (4.0 g./kg) increased the incidence
31   of foci. Co-exposure of methionine (4.0 or 8.0 g/kg) with 3.2 g/l DCA was reported to decrease
32   by ~ 25% DCA-induced glycogen accumulation, increase mortality, but not to have much of an
33   effect on peroxisome enzyme activity (which was not elevated by more than 33% over control for
34   DCA exposure alone). Methionine treatment alone at the 8 g/kg level was reported to increase
35   liver weight, decrease lauroyl-CoA activity and to increase DNA methylation. The authors
36   suggested that their data indicate that methionine treatment slowed the progression of foci to
37   tumors. Given that increasing hypomethylation is associated with tumor progression, decreased
38   hypomethylation from large doses of methionine are consistent with a slowing of progression.
39   Whether, these results would be similar for lower concentrations of DCA and lower
40   concentrations of methionine that were administered to mice for longer durations of exposure,
41   cannot be ascertained from this data. It is possible that in a longer-term study, the number of
42   tumors would be similar. Whether, methionine treatment co-exposure had an effect on the
43   phenotype of foci and tumors was not presented by the authors in this study. Such data would

1 have been valuable to discern if methionine co-exposure at the 4.0 mg/kg level that resulted in an
2 increase in DCA-induce foci, resulted in foci of a differing phenotype or a more heterogeneous
3 composition than DCA treatment alone. Finally, a decrease in tumor progression by methionine
4 supplementation is not shown to be a specific event for the MOA for DCA-induced liver
5 carcinogenicity.
6
7   Tao et al. (2000) reported that 7 days of gavage dosing of TCE (1000 mg/kg in corn oil),
8 TCA (500 mg/kg, neutralized aqueous solution), and DCA (500 mg/kg, neutralized aqueous
9 solution) in 8-week old female B6C3F1 mice resulted in not only increased liver weight but also
10 increased hypomethylation of the promoter regions of c-Jun and c-Myc genes in whole liver DNA
11 (data shown for 1-2 mice per treatment). Treatment with methionine was reported to abrogate this
12 response only at a 300 mg/kg i.p. dose with 0-100 mg/kg doses of methionine having no effect.
13 Ge et al. (2001b) reported DCA- and TCA-induced DNA hypomethylation and cell proliferation
14 in the liver of female mice at 500 mg/kg and decreased methylation of the c-*myc* promoter region
15 in liver, kidney and urinary bladder. However, increased cell proliferation preceded
16 hypomethylation. Ge et al (2002) also reported hypomethylation of the c-*myc* gene in the liver
17 after exposure to the peroxisome proliferators 2,4-dichlorophenoxyacetic acid (2,4-D)(1,680
18 ppm), dibutyl phthalate (DBP) (20,000 ppm), Gemfibrozil (8,000 ppm), and Wy-14,643 (50-500
19 ppm, with no effect at 5 or 10 ppm) after six days in the diet. Caldwell and Keshava (2006)
20 concluded that hypomethylation did not appear to be a chemical-specific effect at these
21 concentrations. As noted above in section 3.3.5, chemical exposure to a number of differing
22 carcinogens have been reported to lead to progressive loss of DNA methylation..
23
24   Caldwell and Keshava (2006) also note similar changes in methylation after initiation and
25 treatment with DCA or TCA. "After initiation by N-methyl-N-nitrosourea (25 mg/kg) and
26 exposure to 20 mmL/L DCA or TCA (46 weeks), Tao et al. (2004) report similar hypomethylation
27 of total mouse liver DNA by DCA and TCA with tumor DNA showing greater hypomethylation.
28 A similar effect was noted for region-2 (DMR-2) of the insulin-like growth factor-II (IGF-II)
29 gene. The authors suggest that hypomethylation of total liver DNA and the IGF-II gene found in
30 non-tumorous liver tissue would appear to be the result of a more prolonged activity and not cell
31 proliferation, while hypomethylation of tumors could be an intrinsic property of the tumors. Over
32 expression of IGF-II gene in liver tumors and preneoplastic foci has been shown in both animal
33 models of hepatocarcinogenesis and humans, and may enhance tumor growth, acting via the over-
34 expressed IGF-I receptor (Scharf et al., 2001; Werner and Le Roith, 2000). IGF-I is the major
35 mediator of the effects of the growth hormone; it thus has a strong influence on cell proliferation
36 and differentiation and is a potent inhibitor of apoptosis (Furstenberger et al., 2002). Normally,
37 expression of IGF-II in liver is greater during the fetal period than the adult, but is over-expressed
38 in human hepatocarcinomas due to activation of fetal promoters (Scharf et al., 2001) and loss of
39 imprinting (Khandawala et al., 2000). Takeda et al. (1996) report IGF-II expression in the liver is
40 monoallelic (maternally imprinted) in the fetal period is relaxed during the postnatal period,
41 (resulting in biallelic expression), and is imbalanced in human hepatocarcinomas (leading to
42 restoration of monoallelic IG-II expression)."

1   However, Bull (2004) and Bull et al. (2004) have recently suggested that hypomethylation
2   and peroxisome proliferation occur at higher exposure levels than those that induce liver tumors
3   for TCE and its metabolites. They report that a direct comparison in the no-effect level or low-
4   effect level for induction of liver tumors in the mouse and several other endpoints shows that, for
5   TCA, liver tumors occur at lower concentrations than peroxisome proliferation *in vivo* but that
6   PPARα activation occurs at a lower dose than either tumor formation or peroxisome proliferation.
7   A similar comparison for DCA shows that liver tumor formation occurs at a much lower exposure
8   level than peroxisome proliferation, PPARα activation, or hypomethylation. In addition, they
9   report that these chemicals are effective as carcinogens at doses that do not produce cytotoxicity.
10
11   *3.4.2.3. Oxidative Stress*
12
13   Several studies have attempted to study the possible effects of "oxidative stress" and DNA
14   damage resulting from TCE exposures. The effects of induction of metabolism by TCE, as well
15   as through co-exposure to ethanol, have been hypothesized to in itself increase levels of "oxidative
16   stress" as a common effect for both exposures (see Section 4.2.4. below). Oxidative stress has
17   been hypothesized to be the MOA for peroxisome proliferators as well, but has been found to
18   neither be correlated with cell proliferation nor carcinogenic potency of peroxisome proliferators
19   (see Section 3.4.1.1). As a MOA, it is not defined or specific as the term "oxidative stress" is
20   implicated as part of the pathophysiologic events in a multitude of disease processes and is part of
21   the normal physiologic function of the cell and cell signaling.
22
23   In regard to measures of oxidative stress, Rusyn et al. (2006) noted that although an
24   overwhelming number of studies draw a conclusion between chemical exposure, DNA damage,
25   and cancer based on detection of 8-hydroxy-2' deoxyguanosine (8-OHdG), a highly mutagenic
26   lesion, in DNA isolated from organs of *in vivo* treated animals, a concern exists as to whether
27   increases in 8-OHdG represent damage to genomic DNA, a confounding contamination with
28   mitochondrial DNA, or an experimental artifact. As described in Section 2.2.8., the study by
29   Channel et al (1998) demonstrated that corn oil as vehicle had significant effects on measures of
30   "oxidative stress" such as thiorbarbiturate acid-reactive substances (TBARS). Also as noted
31   previously (Sections 2.1.1. and 2.2.11.), studies of TCE which employ the i.p. route of
32   administration can be affected by inflammatory reactions resulting from that routes of
33   administration and subsequent toxicity that can involve oxygen radical formation from
34   inflammatory cells.
35
36   The issues with interpretation of the Channel et al (1998) study of TCE administered via
37   corn oil gavage to mice have already been discussed in Section 2.1.7 above. The TBARS results
38   indicated suppression of TBARS with increasing time of exposure to corn oil alone with data
39   presented in such a way for 8-OHdG and total free radical changes that the pattern of corn oil
40   administration was obscured. It was not apparent from that study that TCE exposure induced
41   oxidative damage in the liver.

1   Toraason et al. (1999) measured 8-OHdG and a "free radical-catalyzed isomer of

2   arachidonic acid and marker of oxidative damage to cell membranes, 8-Epi-prostaglandin F2α

3   (8epiPGF)", excretion in the urine and TBARS (as an assessment of malondialdehyde and marker

4   of lipid peroxidation) in the liver and kidney of male Fischer rats (150-200 g) exposed to single 0,

5   100, 500, or 1000 mg/kg TCE i.p. injections in Alkamuls vehicle (n= 6/group). Two sequential

6   urine samples were collected 12 hours after injection and animals were sacrificed at 24 hours with

7   DNA collected from liver tissues and TBARS measured in liver homogenates. The mean body

8   weights of the rats were reported to vary by 13% but the liver weights varied by 44% after the

9   single treatments of TCE (i.e., in contrast to the large volume of the literature that reports TCE-

10   induced increases in liver weight, the 500 and 1000 mg/kg exposed rats were reported to have

11   reduced liver weight by 44% in comparison to the control values). Using this paradigm, 500

12   mg/kg TCE was reported to induce stage II anesthesia and a 1000 mg/kg TCE to induce level III

13   or IV (absence of reflex response) anesthesia and burgundy colored urine with 2/6 rats at 24 hours

14   comatose and hypothermic. The animals were sacrificed before they could die and the authors

15   suggested that they would not have survived another 24 hours. Thus, using this paradigm there

16   was significant toxicity and additional issues related to route of exposure. Urine volume declined

17   significantly during the first 12 hours of treatment and while water consumption was not

18   measured, it was suggested by the authors to be decreased due to the moribundity of the rats.

19   Given that this study examined urinary markers of "oxidative stress" the effects on urine volume

20   and water consumption, as well as the profound toxicity induced by this exposure paradigm, limit

21   the interpretation of the study. The authors noted that because both using volume and creatinine

22   excretion were affected by experimental treatment, urinary excretion of 8-OHdG changed

23   significantly based on the mode of data expression. Excretion of 8epiPGF was reported to be no

24   different from controls 12-24 hours and decreased 24 hours after TCE exposure at the two highest

25   levels. Excretion of 8-OHdG was reported to not be affected by any exposure level of TCE and if

26   expressed on the basis of 24- hours decreased. TBARS concentration per gram of liver was

27   reported to be increased at the 500 and 1000 mg/kg TCE exposure levels (~ 2-3) fold. The effects

28   of decreased liver size in the treated animals for this measure in comparison to control animals,

29   was not discussed by the authors.   For 8-OHdG measures in the liver and lymphocytes, the

30   authors reported that "cost prohibited analysis of all of the tissues samples" so that a subset of

31   animals was examined exhibiting the highest TBARS levels. The number of animals used for this

32   determination was not given nor the data except for 500 mg/kg TCE exposure level. TCE was

33   reported to increase 8-OHdG/dG in liver DNA relative to controls to about the same extent in

34   lymphocytes from blood and liver (~ 2-fold) with the results for liver reported to be significant.

35   The issues of bias in selection of the data for this analysis, as well as the issues already stated for

36   this paradigm limit interpretation of this data while the authors suggest that evidence of oxidative

37   damage was equivocal.

38

39   DCA and TCA have also been investigated using similar measures. Larson and Bull

40   (1992) exposed male B6C3F1 mice [26 ± 3 g (SD)]to a single dose of 0, 100, 300, 1000, or 2000

41   mg/kg/day TCA or 0, 100, 300, or 1000 mg/kg/day DCA in distilled water by oral gavage (n= 4).

42   Fischer 344 rats (237 ± 4 g) received a single oral dose of 0, 100 or 1000 mg/kg DCA or TCA (n=

43   4 or 5) TBARS was measured from liver homogenates and assumed to be malondialdehyde. The

1   authors stated that a preliminary experiment had shown that maximal TBARS was increased 6
2   hours after a dose of DCA and 9 hours after a dose of TCA in mice (data shown) and that by 24
3   hours TBARS concentrations had declined to control values (data not shown). However, time-
4   course information in rats was not presented and the same times used for both species, (i.e., 6- and
5   9- hours time periods after administration of DCA and TCA) for examination of TBARS activity.
6   A dose of 100 mg/kg DCA (rats or mice) or TCA (mice) did not elevate TBARS concentrations
7   over that of control liver with this concentration of TCA not examined in rats. For TCA, there
8   was a slight dose-related increase in TBARS over control values starting at 300 mg/kg in mice
9   (i.e., 1.68-fold, 2.02- –fold, and 2.70-fold of control for 300, 1000, and 2000 mg/kg TCA). For
10   DCA there were similar increases over control for both the 300 and 1000 mg/kg dose levels in
11   mice (i.e., 3.22-fold and 3.45-fold of control, respectively). For rats the 1000 mg/kg and 2000
12   mg/kg levels of TCA were reported to show a statistically significant increase in TBARS over
13   control (i.e., 1.67-fold and 2.50-fold, respectively) with the 300 and 1000 mg/kg level of DCA
14   showing similar increases but with only the 300 mg/kg-induced change statistically significant
15   different than control values (i.e., 3.0-fold and 2.0-fold of control, respectively).   Of note, is the
16   report that the induction of TBARS in mice is transient and has subsided within 24 hours of a
17   single dose of DCA or TCA, that the response in mice appeared to be slightly greater with DCA
18   than TCA at similar doses, and that for DCA, there was similar TBARS induction between rats
19   and mice at similar dose levels.
20
21   A study by Austin et al. (1996) appears to a follow-up publication of the preliminary
22   experiment cited in Larson and Bull (1992). Male B6C3F1 mice (8 weeks old) were treated with
23   single doses of DCA or TCA in buffered solution (300 mg/kg) with liver examined for 8-OHdG.
24   The authors stated that in order to conserve animals, controls were not employed at each time
25   point. For DCA the time course of 8-OHdG was studied at 0, 4, 6, and 8 hours after
26   administration and for TCA at 0, 6, 8, and 10 hours after of a 300 mg/kg dose (n= 6). There was a
27   statistically significant increase over controls in 8-OHdG for the 4- and 6-hour time points for
28   DCA (~ 1.4-fold and 1.5-fold of control, respectively) but not at 8 hours in mice. For TCA, there
29   was a statistically significant increase in 8-OHdG at 8 and 10 hours for TCA (~ 1.4 and 1.3-fold of
30   control, respectively).
31
32   The results for PCO and liver weight for Parrish et al. (1996) are discussed in Section
33   2.3.2.2 above for male B6C3F1 mice exposed to TCA or DCA (0, 0.01, 0.5 and 2.0 g/l) for 3 or 10
34   weeks (n= 6).   The study focused on an examination of the relationship with measures of
35   peroxisome proliferation and oxidative stress. The dose-related increase in PCO activity at 21
36   days (~ 1.5-fold, 2.2-fold, and ~ 4.1-fold of control, for 0.1, 0.5, and 2.g/l TCA) was reported to
37   not be increased similarly for DCA. Only the 2.0 g/l dose of DCA was reported to induce a
38   statistically significant increase at 21-days of exposure of PCO activity over control (~ 1.8-fold of
39   control). After 71 days of treatment, TCA induced dose-related increases in PCO activities that
40   were ~ twice the magnitude as that reported at 21 days (i.e. ~ 9-fold greater at 2.0 g/l level).
41   Treatments with DCA at the 0.1 and 0.5 g/l exposure levels produced statistically significant
42   increase in PCO activity of ~ 1.5-fold and 2.5-fold of control, respectively. The administration of

1.25 g/l clofibric acid in drinking water, used as a positive control, gave ~ 6-7-fold of control PCO activity at 21 and 71 days exposure.

Parrish et al (1996) reported that laurate hydroxylase activity was reported to be elevated significantly only by TCA at 21 days and to approximately the same extent (~ 1.4 to 1.6-fold of control) increased at all doses tested. At 71 days both the 0.5 and 2.0 g/l TCA exposures induced a statistically significant increase in laurate hydroxylase activity (i.e., 1.6-fold and 2.5-fold of control, respectively) with no change reported after DCA exposure. The actual data rather than % of control values were reported for laurate hydroxylase activity with the control values varying 1.7-fold between 21 and 71 days experiments. Levels of 8-OHdG in isolated liver nuclei were reported to not be altered from 0.1, 0.5, or 2.0 g/l TCA or DCA after 21 days of exposure and this negative result was reported to remain even when treatments were extended to 71 days of treatment. The authors noted that the level of 8-OHdG increased in control mice with age (i.e., ~ 2 fold increase between 71-day and 21-day control mice). Clofibric acid was also reported not to induce a statistically significant increase of 8-OHdG at 21 days, but to produce an increase (~ 1.4-fold of control) at 71 days. Thus, the increases in PCO activity noted for DCA and TCA were not associated with 8-OHdG levels (which were unchanged) and also not with changes laurate hydrolase activity observed after either DCA or TCA exposure. Of note is the variability in both baseline levels of PCO and laurate hydrolase activity. Also of note, is that the authors report taking steps to minimize artifactual responses for their 8-OHdG determinations. The authors concluded that their data does not support an increase in steady state oxidative damage to be associated with TCA initiation of cancer and that extension of treatment to time periods sufficient to insure peroxisome proliferation failed to elevate 8-OHdG in hepatic DNA. The increased 8-OHdG at 10 weeks after Clofibrate administration but lack of 8-OHdG elevation at similar levels of PCO induction by were also noted by the authors to suggest that peroxisome proliferative properties of TCA were not linked to oxidative stress or carcinogenic response.

As noted above for the study of Leakey et al (2003a) (Section 2.3.4.), hepatic malondialdehyde concentration in ad libitum fed and dietary controlled mice did not change with chloral hydrate (CH) exposure at 15 months but the dietary controlled groups were all ~ half that of the ad libitum-fed mice. Thus, while overall increased tumors observed in the ad libitum diet correlated with increased malondialdehyde concentration, there was no association between CH dose and malondialdehyde induction for either diet.

4. Effects of co-exposures on MOA – internal and external exposures to mixtures including alcohol

Caldwell et al. (2008b) recently published a review of the issues and studies involved with the effects of co-exposures to TCE metabolites that could be considered internal (i.e., an internal co-exposure for the liver) and co-exposures to metabolites and other commonly occurring chemicals that are present in the environment. As they stated:

1   Human exposure to a pollutant rarely occurs in isolation. EPA's Cumulative Exposure
2   project and subsequent National Air Toxics Assessment have demonstrated that
3   environmental exposure to a number of pollutants, classified as potential human
4   carcinogens, is widespread [U.S. EPA, 2006;Woodruff et al., 1998]. Interactions between
5   carcinogens in chemical mixtures found in the environment have been a concern for
6   several decades. Furthermore, how these interactions affect the mode of action (MOA)
7   by which these chemicals operate and how such effects may modulate carcinogenic risk is
8   of concern as well. Thus, an understanding of the MOA(s) of a pollutant can help
9   elucidate its potential carcinogenic risk to humans, and can also help identify susceptible
10   subpopulations through their intrinsic factors (e.g., age, gender, and genetic
11   polymorphisms of key metabolic and clearance pathways) and extrinsic factors (e.g. co-
12   exposures to environmental contaminants, ethanol consumption, and pharmaceutical use).
13   Trichloroethylene (TCE) can be a useful example for detailing the difficulties and
14   opportunities for investigating such issues because, for TCE, there is both internal
15   exposure to a "chemical mixture" of multiple carcinogenic metabolites [Chiu et al.,
16   2006a, b] and co-exposures from environmental contamination of TCE metabolites, and
17   from pollutants that share common metabolites, metabolic pathways, MOAs, and targets
18   of toxicity with TCE.
19
20   Typically, ground water or contaminated waste sites can have a large number of
21   pollutants that vary in regard to information available to support the characterization of
22   their potential hazard, and that have differing MOAs and targets. For example,
23   Veeramachaneni et al. (2001) reported reproductive effects in male rabbits, resulting from
24   exposure to drinking water containing concentrations of chemicals typical of ground
25   water near hazardous waste sites. The drinking water exposure mixture contained
26   arsenic, chromium, lead, benzene, chloroform, phenol, and TCE. Even at 45 weeks after
27   the last exposure, mating desire/ability, sperm quality, and Leydig cell function were
28   subnormal. However, while the exposure levels are relevant to human environmental
29   exposures, design of this study precludes a conclusion as to which individual toxicant, or
30   combination of the seven toxicants, caused the effects. Thus, this study exemplifies he
31   problems associated with studying a multi-mixture milieu. Studies of the interactions of
32   TCE metabolites or common co-exposures that report the interactions of 2 or 3 chemicals
33   at one time are easier to interpret.
34
35   Since EPA published its 2001 draft assessment, several approaches have been reported
36   that include examination of tumor phenotype, gene expression, and development of
37   physiologically-based pharmacokinetic (PBPK) models to assess possible effects of co-
38   exposure. They attempt to predict whether such co-exposures would produce additivity
39   of response or if co-exposure would change the nature of responses induced by TCE or its
40   metabolites. In addition, new studies on co-exposure to DBA may help identify a co-
41   exposure of concern. These studies may give potential insights into possible MOAs and
42   modulators of TCE toxicity. More recent information on the toxicity of individual
43   metabolites of TCE [Caldwell and Keshava, 2006] may be helpful in trying to identify

which are responsible for TCE toxicity, but may also identify the effects of environmental co-exposures.

Recently, EPA sought advice from the National Academy of Sciences (NAS) [Chiu et al., 2006a] with the NAS charge questions including the following. (1) What TCE metabolites, or combinations of metabolites, may be plausibly involved in the toxicity of TCE? (2) What chemical co-exposures may plausibly modulate TCE toxicity? (3) What can be concluded about the potential for common drinking water contaminants such as other solvents and/or haloacetates to modulate TCE toxicity? (4) What can be concluded about the potential for ethanol consumption to modulate TCE toxicity? Thus, the understanding of the effects of co-exposure, in the context of MOA, is an important element in understanding the risk of a potential human carcinogen.

U.S. EPA's draft TCE risk assessment [U.S. EPA, 2001] identified several factors involving co-exposure to TCE metabolites, environmental contaminants, and ethanol that could lead to differential sensitivity to TCE toxicity. Research needs identified there, as well as in previous reviews [Bull, 2000; Pastino et al., 2000], included further elucidation of the interaction of TCA and DCA in TCE-induced liver tumors and a better understanding of the functional relationships among risk factors. The complexity of TCE's potential interactions with chemical co-exposures from either common environmental co-contaminants or common behaviors such as alcohol consumption mirrors the complexity of the metabolism and the actions of TCE metabolites. Thus, TCE presents a good case study for further exploration of the effects of co-exposure on MOA.

The following Sections first reiterates the findings of Bull et al (2002) in regard to simple co-exposures of DCA and TCA which can be experienced as an internal co-exposure after TCE exposure. A number of studies have examined the effects of TCE or its metabolites after previous exposure to presumably genotoxic carcinogen to not only determine the effect of the co-exposure on liver carcinogenicity but also to use such paradigms to distinguish between the effects of TCA and DCA. Finally, not only is TCE a common co-exposure with its own metabolites, but is also a common co-exposure with other solvents, and the brominated analogues of TCA and DCA. The available literature is examined for potential similarities in target and effects that may cause additional concern. The effects of ethanol on TCE toxicity is examined as well as the potential pharmacokinetic modulation of risk using recently published reports of physiologically based pharmacokinetic (PBPK) models that may be useful in predicting co-exposure effects.

4.1. Internal Co-exposures to TCE Metabolites: Modulation of Toxicity and Implications for TCE MOA

Exposure to TCE will produce oxidative metabolites in the liver as an internal co-exposure. As stated above, the phenotypic analysis of TCE-induced tumors have similarities to combinations of DCA and TCA and in some reports to more closely resemble DCA-induce

1   tumors in the mouse. Results from Bull et al. (2002) are presented in Section 2.2.22. for the
2   treatment of mice to differing concentrations of DCA and TCA in combination and the
3   resemblance of tumor phenotype to that of TCE.   In regard to cancer dose-response, the most
4   consistent treatment-related increase in response occurred with combinations of exposure to DCA
5   and TCA that appeared to increase lesion multiplicity when compared to effects from individual
6   chemicals separately. Bull et al. (2002) presented results for "selected" lesions examined for
7   pathology characterization that suggest co-exposure of 0.5 g/l DCA with either 0.5 g/l or 2 g/l
8   TCA had a greater than additive effect on the total number of hyperplastic nodules. In addition
9   co-exposure to 0.1 g/l DCA and 2 g/l TCA was reported to have a greater than additive effect on
10 the total number of adenomas, but not carcinomas, induced. The random selection of lesions for
11 the determination of potential treatment-related effects on incidence and multiplicity, rather than
12 characterization of all lesions, increases the uncertainty in this finding.
13
14 ## 4.2.   Initiation Studies as Co-exposures
15
16   There is a body of literature that has focused on the effects of TCE and its metabolites
17 after rats or mice have been exposed to "mutagenic" agents to "initiate" hepatocarcinogenesis.
18 Given that most of these "initiating agents" have many effects that are not only mutagenic but
19 also on epigenetic, that the dose and exposure paradigm modify these effects, that "initiators"
20 they can increased tumor responses alone, and the tumors that arise from these protocols are
21 reflective of simultaneous actions of both "initiator" and "promoter," paradigms that first expose
22 rats or mice to a "mutagen" and then to other carcinogenic agents can be described as a co-
23 exposure protocols. As stated previously, DEN and N-nitrosomorpholine have been reported to
24 increase differing populations of mature hepatocytes with DEN not only being a mutagen but
25 also to induce concurrent hepatocyte regeneration at a high dose. Thus, the effects of the TCE or
26 its metabolites are hard to discern from the effects of the "initiating" agent in terms of MOA. As
27 demonstrated in the studies of Pereira et al. below, the gender also determines the nature of the
28 tumor response using these protocols.   In addition, when the endpoint for examination is tumor
29 phenotype the consequences of tumor progression are hard to discern from the MOA of the
30 agents when differing concentrations, different durations of exposure, lesions counted as
31 "tumors" included different stages of tumor progression (foci to carcinoma), and highly variable
32 and low numbers of animals are examined. However, differences in phenotype of tumors
33 resulting from such co-exposures, like the co-exposure studies cited above for co-exposures of
34 TCE metabolites, can help discern that exposure to TCE metabolites results in differing actions
35 as demonstrated by differing effects in the presence of co-carcinogens. As stated above, Kraupp-
36 Grasl et al. (1990) note differences among PPARα agonists in their ability to promote tumors
37 suggest they should not necessarily be considered a uniform group.
38
39 ### 4.2.1. Herren-Freund et al. 1987
40
41   The results of TCE exposure alone were reported previously (2.2.17) for this study. This
42 study's focus was on the effect of TCE, TCA, DCA and Phenobarbital on hepatocarcinogenicity
43 in male B6C3F1 mice after "initiation" at 15 days with 2.5 or 10 μg/g body weight of

1   ethylnitrosourea (ENU) and then subsequent exposure to TCE and other chemicals in drinking
2   water begging at 4 weeks of age (an age when the liver is already undergoing rapid growth).
3   DCA and TCA were given in buffered solutions and sodium chloride given in the water of
4   control animals. The experiment was reported to be terminated at 61 weeks because the "mice
5   started to exhibit evidence of tumors." Concentrations of TCE were 0, 3 and 40 mg/l, of DCA
6   and TCA 0, 2 and 5 g/l, and of Phenobarbital 0 and 500 mg/l. The number of animals examined
7   in each group ranged from 16 to 32. ENU alone in this paradigm was reported to induce
8   statistically significant increases in adenomas and hepatocellular carcinomas (39% incidence of
9   adenomas and 39% incidence of carcinomas vs. 9% and 0% for controls) at the 10 μg/g dose
10   (n=23), but not at 2.5 μg/g dose (n= 22). The effects of high doses of DCA and TCA alone have
11   already been discussed for other studies, as well as the lack of statistical power using a paradigm
12   with so few and variable numbers of animals, the limitations of an abbreviated duration of
13   exposure which does not allow for full expression of a carcinogenic response, and problems of
14   volatilization of TCE in drinking water. DCA and TCA treatments at these levels (5 g/l) were
15   reported to increase adenomas and carcinomas irrespective of ENU pretreatment and to ~ the
16   same extent with and without ENU. TCE at the highest dose was reported to increase the number
17   of animals with adenomas (37% vs. 9% in control) and carcinomas (37% vs. 0% in controls) but
18   only the # of adenomas/animal was statistically significant as the number of animals examined
19   was only 19 in the TCE group. Phenobarbital was reported to have no effect on ENU tumor
20   induction using this paradigm.
21
22   **4.2.2. Parnell et al. 1986**
23
24   This study used a rat liver foci bioassay (γ-glutamyltranspeptidase, i.e., GGT) for hepatic
25   foci after at 3 and 6 month using protocols that included partial hepatectomy, DEN (10 mg/kg) or
26   TCA (1500 ppm in drinking water) treatment, and then promotion with 5000 ppm TCA (i.e., 5
27   g/l) for 10, 20, or 30 days and phenobarbital (500 ppm) in male Sprague-Dawley rats (5-6 weeks
28   old at partial hepatectomy).   The number of animals per group ranged from 4-6. PCO activities
29   were given for various protocols involving partial hepatectomy, DEN, TCA and Phenobarbital
30   treatments but there was no controls values given that did not have a least one of these
31   treatments. Overall it appeared there was a slight decrease of PCO activity in rats treated with
32   partial hepatectomy/DEN/Phenobarbital treatments and a slight increase over other treatments for
33   rats treated with partial hepatectomy/DEN/ 5000 ppm TCA or just TCA from 2 weeks to 6
34   months of sampling. In regard to GGT-positive foci, the partial hepatectomy/DEN/Phenobarbital
35   group (n= 6) was reported to have more positive foci at 3 or 6 months than rats "initiated" with
36   TCA and PB after partial hepatectomy or partial hepatectomy/Phenobarbital treatment alone
37   (2.05 foci/cm^2 vs. ~ .05 – 0.10 foci/cm^2 for all other groups). The number of GGT positive foci
38   in rats without any treatment were not studied or presented by the authors. For "promotion"
39   protocols the number of GGT positive foci induced by the partial
40   hepatectomy/DEN/Phenobarbital protocol at 3 and 6 months, appeared to be reduced when
41   Phenobarbital exposure was replaced by TCA co-exposure but there was no dose-response
42   between the 50, 500 and 5000 ppm. However, TCA treatment along with partial hepatectomy
43   and DEN treatment did increase the levels of foci (means of 0.71 – 0.39 foci/ cm^2 at 3 months

1 and 1.83-2.45 foci/cm^2 at 6 months) over treatment of just partial hepatectomy and DEN (0.05 ±
2 0.20 foci/ cm^2 at 3 months and 0.30 ± 0.39 foci/ cm^2 at 6 months). For the TCA animals treated
3 only with 5000 ppm TCA, the number of GGT positive foci at 3 months was 0.23 ± 0.16 foci/
4 cm^2 and at 6 months 0.03 ± 0.32 foci/ cm^2 with no values for untreated animals presented. For
5 the positive control (partial hepatectomy/DEN/Phenobarbital) the number of GGT positive foci
6 increased from 3 to 6 months (1.65 ± 0.23 foci/ cm^2 and at 6 months 7.61 ± 0.72 foci/ cm^2). The
7 authors concluded that "although TCA is reported to cause hepatic peroxisomal stimulation in
8 rats and mice, the results of this study indicate that it is unlikely TCA's effects are related to the
9 promoting ability seen here. The minimal stimulation of , 10 to 20% over controls of
10 peroxisomal associated, PCO activity in TCA exposed rats was seen only at the 5000 ppm level
11 and only within the promotion protocol. This finding is in contrast to the promoting activity seen
12 at all three concentrations of TCA."
13
14 ### 4.2.3. Pereira and Phelps 1996
15
16 The results for mice that were not "initiated" by exposure to MNU, but exposed to DCA
17 or TCA, are discussed in Section 2.3.2.6. However, differences in responses after initiation are
18 useful for showing differences between single and co-exposures as well as differences between
19 DCA and TCA effects. On day 15 of age, female B6C3F1 mice received an i.p. injection of
20 MNU (25 mg/kg) and at 7 weeks of age received DCA (2.0, 6.67, or 20 mmol/l), TCA (2.0, 6.67
21 mmol, or 20 mmol/l), or NaCl continuously for 31 or 51 weeks of exposure. The number of
22 animals studied ranged from 6 to 10 in 31-week groups and 6 to 39 in the 52-week groups.
23 There was a "recovery group" in which mice received either 20 mmol/l DCA (2.58 g/l DCA)
24 (n= 12) or TCA (3.27 g/l TCA) (n= 11) for 31 weeks and then switched to saline for 21 weeks
25 until sacrifice at 52 weeks. Strengths of the study included the reporting of hepatocellular
26 lesions as either foci, adenomas, or carcinomas and the presentation of incidence and
27 multiplicity of each separately reported for the treatment paradigms. Limitations included the
28 low and variable number of animals in the treatment groups.
29
30 MNU was reported to not "significantly" induce foci or altered hepatocytes, adenomas,
31 or carcinomas at 31 (n= 10) or 51 weeks (n= 39). However, MNU did increase the incidence
32 and number/mouse of foci, adenomas and carcinomas at the 52 week sacrifice time in
33 comparison to saline controls, albeit at lower levels than observed in DCA or TCA co-
34 treatments groups (e.g., 10% vs. 0% foci, 17.5% vs. 2.5% adenomas, and 10% vs. 0% incidence
35 of carcinomas at 52 weeks for MNU-treated mice vs. saline control). Co-exposure of DCA
36 (20.0 mmol/l) for 52 weeks in MNU-treated mice increased the number of foci and
37 hepatocellular adenomas with the authors reporting "the yield of total lesions/mouse increased
38 as a second order function of the concentration of DCA (correlation coefficients ≥ 0.998)."
39 TCA co-exposure in MNU-treated mice was reported to not result in a significant difference in
40 yield of foci or altered hepatocytes with either continuous 52 week or 31-week exposure, but
41 exposures to 20.0 or 6.67 mmol/l TCA did result in increased yield of liver tumors with both
42 exposure protocols (see below).
43

1   For TCA treatment in MNU treated mice, the incidences of foci were similar (12.5% vs.
2   18.2%) but the number of foci/ mouse was ~ 3-fold greater in the cessation protocol than with
3   continuous exposure. The incidence of adenomas was reported to be the same (~ 66%) as well
4   as the number of adenomas/animal between continuous and cessation exposures. For
5   carcinomas, there was a greater incidence for mice with continuous TCA exposure (83% vs.
6   36%) as well as a greater number of carcinomas/mouse (~ 4-fold) than for those initiated mice
7   with cessation of TCA exposure. As noted above, the number of animals treated with TCA was
8   low and variable (e.g., 23 mice studied at 52 weeks 20.0 mmol/l TCA, and 6 mice at 52 weeks
9   6.6.7 mmol/l TCA), limiting the ability to discern a statistically significant effect in regard to
10   dose-response. The concentration- response relationship for tumors/mouse after 31 and 51
11   weeks was reported to be best represented by linear progression.

12

13   A comparison of results for animals treated with MNU and 20.0 mmol/l DCA or TCA
14   for 31 weeks and sacrificed at 31 weeks and those which were treated with MNU and DCA or
15   TCA for 31 weeks and then sacrificed at 52 weeks is limited by the number of animals exposed
16   (n= 10 for 31 week sacrifice DCA or TCA, n= 12 for DCA recovery group, and n= 11 for TCA
17   recovery group). No carcinoma data was reported for animals exposed at 31 weeks and
18   sacrificed at 31 weeks making comparisons with recovery groups impossible for this parameter
19   and thus determinations about progression from adenomas to carcinomas. For the MNU and
20   DCA-treated animals, the incidence or number of animals reported to have foci at 31 weeks was
21   reported to be 80% but 38.5% for in the recovery group. For adenomas the incidence was
22   reported to be 50% for DCA-treated animals at 31 weeks and 46.2% for the recovery group. For
23   MNU and TCA-treated animals, the incidence of foci at 31 weeks was reported to 20% and
24   18.2% for the recovery group. For adenomas the incidence was reported to be 60% for the
25   TCA-treated animals at 31 weeks and 63.6% for the recovery group. Thus, this limited data set
26   shows a decrease in incidence of foci for the MNU and DCA-treated recovery group but no
27   change in incidence of foci for TCA or for adenomas for DCA- or TCA-treatment between
28   those sacrificed at 31 weeks and those sacrificed 21 weeks later. In regard to multiplicity, the
29   number of foci/mouse was reported to be 2.80 ± 0.20 for the 31-week DCA group and $0.46 \pm$
30   0.18 for the recovery group (mean $\pm$ SEM). The number of adenomas/mouse was reported to be
31   1.80 ± 0.83 for the 31-week group and 0.69 ± 0.26 for the recovery group. Thus, both the
32   number of foci and adenomas per mouse was reported to be decreased after the recovery period
33   for MNU and DCA treated mice. Given that the number of animals with foci was decreased by
34   half, the concurrent decrease in foci/mouse is not surprising. For TCA treatments, the numbers
35   of foci/mouse were reported to be 0.20 ± 0.13 for the 31-week group and 0.45 ± 0.31 for the
36   recovery group. The number of adenomas/mouse for TCA-treatment groups was reported to be
37   1.30 ± 0.45 for the 31-week group and 0.91 ± 0.28 for the recovery group. For the MNU and
38   TCA-treated mice the numbers of foci/mouse were reported to be increased and the number of
39   adenomas/mouse reported to be slightly lower. Because carcinoma data is not presented for the
40   31 week group, it is impossible to determine whether the TCA adenomas regressed to foci or the
41   TCA adenomas progressed to carcinomas and more foci apparent with increased time.

1   For the comparison of the numbers of foci, adenomas, or carcinomas per mouse that
2 were reported for the mice exposed at 31 weeks and sacrificed and those exposed for 52 weeks,
3 issues arise as to the impact of such few animals studied at 31 weeks, and the differing
4 incidences of lesions reported for these mice on tumor multiplicity estimates. The number of
5 animals studied who treated with MNU and 20.0 mmol/l DCA or TCA for 31 weeks and then
6 sacrificed was n= 10, while the number of animals exposed to 20.0 mmol/l DCA or TCA for 52
7 weeks was 24 for the DCA group and 23 for the TCA group. The number of animals treated at
8 lower concentrations of DCA or TCA were even lower at the 31-week sacrifice (e.g., n= 6 for
9 MNU and 6.67 mmol/l DCA at 31 weeks) and also for the 52-week durations of exposure (e.g.,
10 n= 6 for MNU and 6.6.7 mmol/l TCA).
11
12   At 31 weeks, 80% of the animals were reported to have foci and 50% to have foci after
13 52 weeks of exposure to 20.0 mmol/l DCA and MNU treatment. Thus, similar to the "recovery"
14 experiment, the number of animals with foci decreased even with continuous exposure between
15 31 and 52 weeks. For adenomas, 20.0 mmol DCA exposure for 31 weeks was reported to
16 induce adenomas in 50% of mice and after 52 weeks of exposure to induce adenomas in 73% of
17 mice. For TCA, the number of animals with foci was reported to be 20% at 31 weeks and 12%
18 at 52 weeks after exposure to 20.0 mmol/l TCA after MNU treatment and similar to the
19 incidence of foci reported for the TCA-recovery group. For 20.0 mmol TCA, adenomas
20 reported in 60% of mice after 31 weeks and in 67% of mice after 52 weeks of exposure and also
21 similar to the incidence of adenomas reported for the TCA-recovery group. In regard to
22 multiplicity, the number of foci/mouse was decreased from 2.80 ± 0.20 to 1.46 ± 0.48 between
23 31 weeks and 52 weeks of 20.0 mmol DCA in MNU exposed mice. The number of
24 adenomas/mouse was reported to be increased from 1.80 ± 0.83 to 3.62 ± 0.70 between 31
25 weeks and 52 weeks of 20.0 mmol DCA and MNU exposed mice. For 20.0 mmol/l TCA, the
26 number of foci/mouse was 0.20 ± 0.13 and 0.13 ± 0.7 for 31- and 52-week exposures. The
27 number of adenomas/mouse was reported to be 1.30 ± 0.45 and 1.29 ± 0.24 for 31- and 52-week
28 exposures. Thus, by only looking at foci and adenoma multiplicity data, there would not appear
29 to be a change between 31 and 52-weeks. However, during progression a shift may occur such
30 that foci become adenomas with time and adenomas become carcinomas with time. For
31 carcinomas there was no data reported for 31 week exposure in MNU and DCA- or TCA-treated
32 mice. However, at 52 weeks 20.0 mmol DCA was reported to induce carcinomas in 19.2% of
33 mice and 20.0 mmol TCA to induce carcinomas in 83% of mice. The corresponding numbers of
34 carcinomas/mouse was 0.23 ± 0.10 for 20.0 mmol/l DCA treatment and 2.79 ± 0.48 for 20.0
35 mmol/l TCA treatment at 52 weeks in MNU treated mice. Thus, although fewer than 20% of
36 MNU-treated mice were reported to have foci at 20.0 mmol TCA, by 52 weeks almost all had
37 carcinomas with ~ 67% also having adenomas. For DCA, many more mice had foci at 31
38 weeks (80%) than for TCA and by 52 weeks ~ 70% had adenoma with only ~ 20% reported to
39 have carcinomas. The incidence data are suggestive that as these high doses of DCA and TCA,
40 TCA was more efficient inducing progression of a carcinogenic response than DCA in MNU-
41 treated mice.
42

1   The authors interpret the decrease in foci and adenomas between animals treated with
2   MNU and 20.0 mmol/l DCA for 31 weeks and sacrificed and those sacrificed 21 weeks later to
3   indicate that these lesions were dependent on continued exposure. However, the total number of
4   lesions cannot be ascertained because carcinoma data was not reported for 31-week exposures.
5   Carcinomas were reported in the recovery group at 52 weeks (0.15 ± 0.10 carcinomas/mouse in
6   15.4% of animals). Of note is that not only did the number of foci/mouse and incidence
7   decrease between the 31-week group and the recovery group, but also between 31- and 52-
8   weeks of continuous exposure for the MNU and 20.0 mmol/l DCA treated groups. Although
9   derived from very few animals, the 6.67 mmol/l DCA group reported no change for foci/mouse
10   but a decrease in the incidence of foci between 31- and 52-weeks of exposure in MNU treated
11   mice (i.e., 0.67 ± 0.18 foci/mouse in 50% of the animals at 31 weeks and 0.50 ± 0.34 foci/mouse
12   in 20% of mice treated for 52 weeks). The numbers of foci/mouse for both MNU-treated and
13   untreated control mice were reported to be decreased between 31 and 51 weeks as well. As
14   noted in Section 3.1.8., the number of "nodules" in humans, which may be analogous to foci and
15   adenomas, can spontaneously regress with time rather than becoming hepatocellular
16   carcinomas. Also as tumors get larger with progression, the number of tumors/mouse can
17   decrease due to coalescence of tumors and difficulty distinguishing between them. While data
18   is suggestive of a decrease in the number of adenomas/mouse after cessation of DCA exposure,
19   the incidence data is similar between the 31-week exposure and recovery groups. Of note is that
20   the number of carcinomas/mouse and the incidence of carcinomas was reported to be similar
21   between the MNU-treated mice exposed continuously to 20.0 mmol/l DCA for 52 weeks and
22   those which were treated for 31 weeks and then sacrificed at 52 weeks. Also of note is that,
23   although incidences and multiplicities of foci and adenomas was reported to be relatively low in
24   the 2.0 mmol/l DCA exposure groups, at 52-weeks 40% of the mice tested had carcinomas with
25   0.70 ± 0.40 carcinomas/mouse. This was a greater percentage of animals with carcinomas and
26   multiplicity than that reported for the highest dose of DCA. This result suggests that the effects
27   in regard to tumor progression, and specifically for carcinoma induction, differ between the
28   lowest and highest doses used in this experiment. However, the low numbers of animals
29   examined for the lower doses, 31-weeks exposures, and in the recovery group decrease the
30   confidence in the results of this study in regard to the effects of cessation of exposure on tumor
31   progression.
32
33   In regard to tumor phenotype, in MNU-treated female mice that were not also exposed
34   to either DCA or TCA, all four foci and 86.7% of 15 adenomas were reported to be basophilic
35   and 13.3 % eosinophilic at the end of the 52 week- study. However, when MNU-treated female
36   mice were also exposed to DCA the number eosinophilic foci and tumors increased with
37   increasing dose after 52 weeks of continuous exposure.. At the 20.0 mmol/l level all 38 foci
38   examined were eosinophilic and 99% of the tumors (almost all adenomas) were eosinophilic.
39   At the 2.0 mmol/l DCA exposure there were no foci examined but about 5 of 9 tumors
40   examined ($\sim$ 2:1 carcinoma:adenoma ratio) were basophilic and the other 4 were eosinophilic.
41   For TCA co-exposure in MNU-treated mice, the 20 mmol/l TCA treatment was reported to give
42   results of 1 of the 3 foci examined to be basophilic and 2 that were eosinophilic. For the 98
43   tumors examined ($\sim$ 2:1 carcinoma/adenoma ratio) 71.4% were reported to be basophilic and

28.6% were eosinophilic. At the 2.0 mmol/l TCA exposure level, the 2 foci examined were reported to be basophilic while the 6 tumors (all adenomas) were reported to be 50% eosinophilic and 50% basophilic.   Thus, after 52 weeks female mice treated with MNU and a high dose of DCA had eosinophilic foci and adenomas and those treated with the high dose of TCA had a mixture of basophilic and eosinophilic foci and tumors with a 3:1 ratio of tumors (mostly carcinomas) being basophilic. At the lower doses of either DCA or TCA the tumors tended to be mostly carcinomas for DCA and adenomas for TCA but both were ~ 50% basophilic and 50% eosinophilic. The tumors observed from MNU treatment alone were all adenomas and mostly 87% basophilic. Thus, not only did treatment concentrations of DCA and TCA give a different result for tumor multiplicity and incidence, but also for tumor phenotype in MNU treated female mice. Eosinophilic foci and tumors were reported to consistently be GST-π positive while basophilic lesions "did not contain GST-π, except for a few scattered cells or very small area comprising less than 5% of the tumor."

Thus, exposure to either DCA or TCA increased incidence and number of animals with lesions (foci, adenomas, or carcinomas) in MNU- vs. non-treated mice (see Section 2.3.2.6 above).   These results suggest that the pattern of foci, adenoma and carcinoma incidence, multiplicity, and progression appeared to differ between TCA and DCA in MNU-treated female mice. However, the low and variable number of animals used in this study, make quantitative inferences between DCA and TCA exposures in "initiated" animals, problematic.

4.2.4. Tao et al. 2000

The source of liver tumors for this analysis was reported to be the study of Pereira and Phelps (1996). Samples of liver "tumors" and "non-involved" liver was homogenized for protein expression for c-Jun and c-Myc and therefore contained homogeneous cell types for study.   The term "liver tumors" was not defined so it cannot be ascertained as to whether the lesions studied were altered foci, hepatocellular adenomas, or carcinomas. Liver tissues were reported to be frozen prior to study which raises issues of m-RNA quality. Although this study reports that there were no MNU-induced "tumors" the original paper of Pereira and Phelps (1996) reports that there were four foci and 15 adenomas in MNU-only treated mice.   The authors reported no difference in c-Jun and c-Myc m-RNA from DCA or TCA-induced tumors from mice "initiated" with MNU.   DNA methyltransferase was reported to be decreased in non-involved liver in MNU-only treated mice in comparison to that from TCA- and DCA-treated mice.   For a comparison between non-involved liver and tumors, tumors were reported to have a greater level than did non-involved liver.

4.2.5. Lantendresse and Pereira 1997

This study used the tumors from Pereira and Phelps (1996), except for the MNU-treated only groups and those groups treated with either DCA or TCA but not MNU initiation, to further study various biomarkers. The omissions were cited as to be due to insufficient tissue. For immunohistochemical evaluation of the molecular biomarkers other than GST-π, liver specimens

1   from 7 MNU/ 20.0 mmol DCA- (i.e. 2.58 g/l DCA) treated and 6 MNU/20.0 mmol TCA - (i.e.,
2   3.27 g/l TCA) treated female mice randomly selected. For GST-π, the number of animals from
3   which lesion specimens were derived, was 24 MNU/DCA-treated and 23 MNU/TCA-treated
4   mice. The DCA treated mice were reported to have 1-9 lesions/mouse and TCA treated mice 1-3
5   lesions/mouse. The number of lesions examined for each biomarker varied greatly. For TCA-
6   induced foci, no foci were examined for any biomarker except 3 lesions for GST-π, while for
7   DCA 12-15 foci were examined for each biomarker and 38 lesions examined for GST-π.
8   Similarly for TCA-induced adenomas, there were 8-10 lesions examined for all biomarkers with
9   32 lesions examined GST-π, while for DCA 12 lesions for all biomarkers with 94 lesions
10   examined for GST-π. Finally, for TCA-induced carcinomas there were 3-4 lesions examined per
11   group with 64 lesions examined for GST-π, while for DCA-induced carcinomas there were no
12   lesions examined for any biomarker except 3 examined for GST-π. The biomarkers used were:
13   GST-π, TGF-α, TGF-β, *c-Jun*, *c-Fos*, *c-Myc*, Cytochrome oxidase CYP2E1, and Cytochrome
14   oxidase CYP4A1.
15
16   MNU/DCA treatment was reported to produce "predominantly eosinophilic lesions" with
17   "in general, the hepatocytes of DCA-promoted foci and tumors were less pleomorphic and
18   uniformly larger and had more distinctive cell borders than the hepatocytes in lesions caused by
19   TCA. Parenchymal hepatocytes of DCA-promoted mice were uniformly hypertrophied, with
20   prominent cell borders, and the cytoplasm was markedly vacuolated, which was morphologically
21   consistent with the previous description of glycogen deposition in these lesions. In contrast,
22   TCA-promoted proliferative lesions tended to be basophilic, as previously reported, and were
23   composed of hepatocytes with less distinct cell borders, slight cytoplasmic vacuolization, and
24   greater variability in nuclear size and cellular size."
25
26   The hepatocytes of altered foci and hepatocellular adenomas from MNU-treated female
27   mice also treated with DCA were reported to stain positively for TGF-α, c-Jun, c-Myc, CYP 2E1,
28   CYP 4A1, and GST-π. The authors do not present the data for foci and adenomas separately but
29   as an aggregate and as the number of lesions with < 50% cells stained or the number of lesions
30   with > 50% cells stained either "minimally to mildly" or "moderately to densely" stained.
31   Because no carcinomas for DCA were examined and especially because no foci for TCA
32   analyses were included in the aggregates, it is difficult to compare the profile between TCA and
33   DCA exposure in initiated animals and to separate these results from the effects of differences in
34   tumor progression. Thus, any differences seen in these biomarkers due to progression from foci
35   to adenoma in DCA-induced lesions or from progression of adenoma to carcinoma in TCA-
36   induce lesions, was lost. If the results for adenomas had been reported separately, there would
37   have been a common stage of progression from which to compare the DCA and TCA effects on
38   initiated female mice liver tumors. For DCA-induced "lesions" (~ 50% foci and ~ 50%
39   adenomas), most lesions had > 50 % cells staining with moderate to dense levels for TGF-α, and
40   CYP2E1, CYP4A1, and GST-π and most lesions had <50% cells staining for even minimally to
41   mild staining for TGF-β and *c-Fos*. For *c-Jun* and *c-Myc* the aggregate DCA-induced "lesions"
42   were heterogeneous in the amount of cells and the intensity of cell staining for these biomarkers
43   in MNU-treated female mice.

1   For the TCA "lesions" (~ 60% adenomas and ~ 30% carcinomas) the authors note that "in
2   general, the hepatocytes of tumors promoted by TCA demonstrated variable immunostaining.
3   With the exception of c-Jun, greater than 50% of the hepatocytes in TCA lesions were essentially
4   negative or stained only minimally to mildly for the protein biomarkers studies. In some
5   instances, particularly in TCA-promoted tumors, there was regional staining variability within
6   the lesions, including immunoreactivity for c-Jun and c-Myc proteins, consistent with clonal
7   expansion or tumor progression." As stated above, the term "lesion" refers to foci and adenomas
8   for DCA but for adenomas and carcinomas for TCA making inferences as to differences in the
9   actions of the two compounds through the comparisons of biomarkers confounded by the effects
10   of tumor progression. The largest differences in patterns between TCA induced "lesions" and
11   those by DCA appeared to be TGF-α (with no lesions having >50% cells stained mildly or
12   moderately/densely for TCA-induced lesions), CYP2E1 (with few lesions having >50% stained
13   moderately/densely for TCA-induced lesions), CYP4A1 (with no lesions having >50% stained
14   mildly or moderately/densely for TCA-induced lesions), and GST-π (with all lesions having <
15   50% cells stained even mildly for TCA-induced lesions). However, as shown by this data, while
16   the "lesions" induced by TCA and DCA had some commonalities within each treatment, there
17   was heterogeneity of lesions produced by both treatments in female mice already exposed to
18   MNU. Overall, the tumor biomarker pattern suggests differences in the effects of DCA and TCA
19   through differences in tumor phenotype they induce as co-exposures with MNU treated female
20   mice.
21
22   The authors note that non-lesion parenchymal hepatocytes in DCA–treated initiated mice
23   stained mostly negative for CYP2E1 and CYP4A1, while in TCA-treated mice staining patterns
24   in parenchymal non-lesions hepatocytes were centrilobular for CYP2E1 and panlobular for CYP
25   4A1 (a pattern for CYP4A1 that is opposite of that found in the TCA-induced lesions).
26

4.2.6. Pereira et al. 1997

28
29   This study used a similar paradigm as that of Pereira and Phelps (1996) to study co-
30   exposures of TCA and DCA to female B6C3F1 mice already exposed to MNU. At 15 days the
31   mice received 25 mg/kg MNU and starting at 6 weeks of age neutralized solutions of either 0,
32   7.8, 15.6, 25.0 mmol/l DCA (n= 30 for control and 25 mmol/l DCA and n=20 for 7.8 and 15.6
33   mmol/l DCA), 6.0 or 25.0 mmol/l TCA (n= 30 for 25.0 mmol/l TCA and n=20 for 6.0 TCA), or
34   combinations of DCA and TCA that included 25.0 mmol/l TCA + 15.6 mmol/l DCA (n=20), 7.8
35   mmol/l DCA + 6.0 mmol/l TCA (n=25), 15.6 mmol/l DCA + 6.0 mmol/l TCA (45), 25.0 mmol/l
36   DCA + 6.mmol/l TCA (n= 25). The corresponding concentrations of DCA and TCA in g/l is 25
37   mmol = 3.23 g/l, 15.6 mmol = 2.01 g/l and 7.8 mmol = 1.01 g/l DCA and 25 mmol= 4.09 g/l and
38   6.0 mmol = 0.98 g/l TCA. Accordingly, the number of animals at the beginning of the study
39   varied between 20 and 45. At terminal sacrifice (after 44 weeks of exposure) the numbers of
40   animals examined were less with the lowest number examined to be 17 mice in the 7.8 mmol/l
41   DCA group and the largest to be 42 in the 15.6 mmol/l DCA + 6.0 mmol/l TCA exposed group.

1   The authors reported that only a total of eight hepatocellular carcinomas were found in the
2   study (i.e., 25.0 mmol/l DCA induced 3 carcinomas, 7.8 mmol DCA + 6.0 mmol TCA induced
3   one carcinoma, and 25.0 mmol/l TCA induced 4 carcinomas). Thus, they presented data for
4   foci/mouse, and adenomas/mouse and their sum of both as "total lesions." The incidences of
5   lesions (i.e., how many mice in the groups had lesions) were not reported. The shortened
6   duration of exposure (i.e., 44 weeks), the omission of carcinomas from total "lesion" counts
7   (precluding consideration of progression of adenomas to carcinomas), the lack of reporting of
8   tumor incidences between groups, and the variable and low numbers of animals examined in
9   each group make quantitative inferences regarding additivity of these treatments difficult. MNU
10   treated mice did have a neoplastic response, albeit low using this paradigm. For mice that were
11   only exposed to MNU (n=30 at terminal sacrifice) the mean number of foci, adenomas and
12   "lesions" per mouse were 0.21, 0.07 and 0.28, respectively. No data were given for mice without
13   MNU treatment but few lesions would be expected in controls. Pereira and Phelps (1996)
14   reported that saline-only treatment in 40 female mice for 51 weeks resulted in 0% foci, 0.03
15   adenomas/mouse in 2.5% of mice, and 0% carcinomas.   In general, it appeared that the numbers
16   of foci, adenomas and the combination of both reported as "lesions" per mouse that would have
17   been predicted by the addition of multiplicities given for DCA, TCA and MNU treatments alone,
18   were similar to those observed as co-exposure treatments. The largest numbers of foci and
19   adenomas/mouse were reported for the 25.0 mmol/l DCA and 6.0 mmol/l TCA treatments in
20   MNU treated mice (mean of 6.57 "lesions"/mouse) with the lowest number reported for 7.8
21   mmol/l DCA and 6 mmol/l TCA (mean of 1.16 "lesions"/mouse).
22
23   The authors reported that the foci of altered hepatocytes were predominantly eosinophilic
24   in DCA-treated female mice initiated with MNU, while those observed after MNU and TCA
25   treatment were basophilic. MNU treatment alone induced 4 basophilic and 2 eosinophilic foci,
26   and 2 basophilic adenomas. MNU and DCA treatment was reported to produce only eosinophilic
27   foci and adenomas at the 25.0 mmol/l DCA exposure level. At the 7.8 mmol/l DCA level of
28   treatment in MNU-treated mice, 2 foci were basophilic, 4 were eosinophilic and the 1 adenoma
29   observed was reported to be eosinophilic. Thus, the concentration of exposure appeared to alter
30   the tincture of the foci observed after MNU and DCA exposure using this paradigm. In this
31   study, MNU and TCA treatment was reported to induce foci and adenomas that were all
32   basophilic at both 25.0 mmol/l TCA and 6.0 mmol/l TCA exposures. After 7.8 mmol/l DCA +
33   6.0 mmol/l TCA exposure, 2/23 foci were basophilic and 21/23 foci were reported to be
34   eosinophilic while all 4 adenomas reported for this group were eosinophilic.
35
36   Irrespective of treatment, eosinophilic foci for were reported to be GST-π positive and
37   basophilic foci to be GST-π negative. An exception was the 4 carcinomas in the group treated
38   with 25 mmol/l TCA which were reported to be predominantly basophilic but contained small
39   areas of GST-π positive hepatocytes.
40
41   It should be noted that the increased dose (up to3.23 g/l DCA and 4/09 g/l TCA) raises
42   issues of toxicity and effects on water consumption as other studies have noted toxicity at highly
43   doses of DCA and TCA. The use of an abbreviated duration of exposure in the study raises

1　issues of sensitivity of the bioassay at the lower doses used in the experiment. In particular, was
2　enough time provided to observe the full development of a tumor response? Finally, a question
3　arises as what can be concluded from the low numbers of foci examined in the study and the
4　affect of such low numbers on the ability to discern differences in these foci by treatment. As
5　with Pereira and Phelps, there appeared to be a difference the nature of the response induced by
6　co-exposure of MNU to relatively high vs. low DCA concentrations. Of note is that while this
7　experiment reported no hepatocellular carcinomas at the lowest dose of DCA at 44 weeks (7.8
8　mmol DCA), Pereira and Phelps (1996) reported that in 9 mice treated with MNU and 2.0 mmol
9　DCA for 52 weeks, there were no foci but 20% of mice had adenomas (0.20 adenomas/mouse)
10　and 40% of mice had carcinomas (0.70 carcinomas/mouse).

12　These results suggest that DCA co-exposure affects TCA-induced lesions. The authors
13　concluded that mixtures of DCA and TCA appear to be at least additive and likely synergistic
14　and similar to the pathogenesis of DCA.

16　**4.2.7. Tao et al. 1998**

18　The focus of this study was an examination of tumors resulting from MNU and DCA or
19　TCA exposure in mice with the source of tumors was reported to be the study of Pereira et al.
20　(1997). Thus, similar concerns discussed above for that study paradigm are applicable to the
21　results of this study. The authors stated that there were also two recovery groups in which
22　exposure was terminated 1 week prior to euthanization at week 44. The Pereira et al (1997)
23　study does not report a cessation group in the study. In this study the number of animals treated
24　in the cessation group, the incidences of tumors in the mice, and the number of tumors examined
25　were not reported. Another group of female B6C3F1 mice (7-8 weeks old) were reported to not
26　be administered MNU but given 25 mmol/l DCA (3.23 g/l DCA), 25 mmol TCA (4.09 g/l TCA),
27　or control drinking water for 11 days (n= 7).

29　Hepatocellular adenomas in DCA-treated mice, adenomas and carcinomas in TCA-treated
30　mice were reported to be analyzed for %-5-methylcytosine in the DNA of tumor tissues. The
31　levels of 5-methylcytosine in liver DNA of mice administered DCA or TCA for 11 days were
32　reported to be reduced in comparison to control tissues (reduced to $\sim$ 36% of control for DCA
33　and $\sim$ 41% of control for TCA with the control value reported to be $\sim$ 3.5% of DNA methylated).
34　The number of animals examined was reported to be 7-10 animals per group.

36　For control liver from mice that had received MNU but not DCA or TCA, and non-
37　involved liver after 44 week of exposure to either, the levels of 5-methylcytosine were similar
38　and not different from the $\sim$ 3.5% of DNA methylated in untreated mice in the 11-days
39　experiment. Thus, initial decreases in methylated DNA shown by exposure to DCA or TCA
40　alone for 11 days, were not observed in "non-involved" liver of animals exposed to either DCA
41　or TCA and MNU.

1   In regard to tumor tissues, the level of 5-methylcytosine in DNA of hepatocellular
2   adenomas receiving DCA and MNU was reported to be decreased by 36% in comparison to
3   noninvolved liver from the same animals. When exposure to DCA was terminated for 1 week
4   prior to sacrifice the level of 5-methylcytosine in the adenomas was reported to be higher and no
5   longer differed statistically from the non-involved liver from the same animal or liver from
6   control animals only administered MNU. The number of samples was reported to be 9-16
7   samples without identification as to how many samples were used for each tumor analysis or how
8   many animals provided the samples (i.e., were most of the adenomas from on animal?)
9
10   For TCA the 5-methylcytosine level was reported to be reduced by 40% in hepatocellular
11   adenomas and 51% reduction in hepatocellular carcinomas in comparison to noninvolved liver
12   from the same animals. These levels were also reported to be less than that the control animals
13   administered only MNU. Termination of exposure to TCA 1 week prior to sacrifice was reported
14   to not produce a statistically significant change in the level of 5-methylcytosine in either
15   adenomas or carcinomas. The levels of 5-methylcytosine were reported to be lower in
16   carcinomas than adenomas (~ 20% reduction) and to be lower in the "recovery" carcinomas than
17   continuous carcinomas (~ 25%) but were not reported as statistically significant. The results are
18   reported to have been derived from 8-16 "samples each". Again information on the number of
19   animals with tumors, whether the tumors were from primarily from one animal, and which DNA
20   results are from 8 vs. 16 samples, was not provided by the authors. Given that Pereira et al.
21   (1997), the source for material of this study, reported that treatment of MNU and 25.0 mmol/l
22   TCA treatment for 44 weeks induced only 4 carcinomas, a question arises as to how many
23   carcinomas were used for the 44-week 5-methylcytosine results in this study for carcinomas (i.e.,
24   how can 8-16 samples arise from 4 carcinomas?). In addition, a question arises as to whether
25   there was a difference in tumor-response in those animals with and without one week of
26   cessation of exposure which cannot be discerned from this report. The use of highly variable
27   number of samples between analysis groups and lack of information as to how many tumors were
28   analyzed adds uncertainty to the validity of these findings. There did not appear to be a
29   difference in methylation activity from short-term exposure to either DCA or TCA alone in
30   whole liver DNA extracts. However, the authors conclude that the difference in methylation
31   status between tumors resulting from MNU and DCA or TCA exposures supports differences in
32   the action between DCA and TCA.
33
34   **4.2.8. Stauber et al. 1998**
35
36   In this study, 5-8 week old male B6C3F1 mice were used for isolation of primary
37   hepatocytes which were subsequently isolated and cultured in DCA or TCA. In a separate
38   experiment 0.5 g/l DCA was given to mice as pretreatment for 2 weeks prior to isolation.   The
39   authors note that and indication of an "initiated cell" is anchorage-independent growth. DCA
40   and TCA solutions were neutralized before use. The primary hepatocytes from 3 mice per
41   concentration were cultured for 10 days with DCA or TCA colonies (8 cells or more) determined
42   in quadruplicate. The levels of DCA used were 0, 0.2, 0.5 and 2.0 mM DCA or TCA. At
43   concentrations of 0.5 mM or more DCA and TCA both induced an increase in the number of

1 colonies that was statistically significant and increased with dose with DCA giving a slightly
2 greater effect. The authors noted that concentrations greater than 2.0 mM were cytotoxic but did
3 not show data on toxicity for this study.
4
5 Of great interest is the time-course experiment from this study in which the number of
6 colonies from DCA treatment *in vitro* peaked by 10 days and did not change through days 15- 25
7 at the highest dose. For the lower concentrations of DCA increased time in culture induced
8 similar peak levels of colony formation by days 20-25 as that reached by 10 days at the higher
9 dose. Therefore, the number of colonies formed was independent of dose if the cells were treated
10 long enough *in vitro*. The number of colonies that formed in control hepatocyte cultures also
11 increased with time but at a lower rate than those treated with DCA (2.0 mM DCA gave ~ 2-fold
12 of control by 25 days of exposure to hepatocytes in culture). However the level reached by cells
13 untreated in tissue culture alone by 20 days was similar to the level induced by 0.5 mM DCA by
14 10 days of exposure. This finding raises the issue of what these "colonies" represent as tissue
15 culture conditions alone transform these cells to what the authors suggest is an "initiated" state.
16 TCA exposure was not tested with time to see if it had a similar effect with time as did DCA.
17
18 At 10 days, colonies were tested for c-Jun expression with the authors noting that
19 "colonies promoted by DCA were primarily c-Jun positive in contrast to TCA promoted colonies
20 that were predominantly c-Jun negative." For colonies that arose spontaneously from tissue
21 culture conditions, 10/13 (76.9%) were reported to be c-Jun +, those treated with DCA 28/34
22 (82.3%) were c-Jun +, and those treated with TCA 5/22 (22.7%) were c-Jun +.   These data show
23 heterogeneity in cell in colonies although more were c-Jun + with DCA than TCA. The number
24 of colonies reported in the c-Jun labeling results represent sums between experiments and thus
25 present total numbers of the control and the of colonies derived from doses of DCA and TCA at
26 0.2 to 2.0 mM at 10 days. Thus, changes in colony c-Jun+ labeling due to increasing dose cannot
27 be determined. The authors reported that with time (24, 48, 72, and 96 hours) of culture
28 conditioning the number of c-Jun+ colonies was increased in untreated controls.   DCA treatment
29 was reported to delay the increase in c-Jun+ expression induced by tissue culture conditions
30 alone in untreated controls. TCA treatment was reported to not affect the increasing c-Jun+
31 expression that increased with time in tissue culture. In this instance, tissue culture environment
32 alone was shown to transform cells and can be viewed as a "co-exposure." DCA pretreatment *in
33 vivo* was reported to increase the number of colonies after plating which reached a plateau at 0.10
34 mM and gave changes as at low a concentration of 0.02mM DCA administered *in vitro*. The
35 background level of colony formation varied between controls (i.e., 2-fold different in
36 pretreatment experiments and non-pretreatment experiments). Therefore although the number of
37 colonies was greater for pretreatment with DCA, the magnitude of difference over the control
38 level was the same after DCA treatment *in vitro* with and without pretreatment.
39
40 The authors presented a comparison of "tumors" from Stauber and Bull (1997) and state
41 that DCA tumors were analyzed after 38 weeks of treatment but that TCA tumors were analyzed
42 after 52 weeks. They note that 97.5% of DCA-induced "tumors" were c-Jun + while none of the
43 TCA-induced "tumors" were c-Jun +.   The concentrations used to give tumors *in vivo* for

1 comparison with *in vitro* results were not reported. What was considered to be "tumors" from
2 the earlier report for this analysis was also not noted. Stauber and Bull (1997) reported results
3 for combination of foci and tumors raising issues as to what was examined in this report. The
4 authors stated that because of such short time, no control tumors results were given. The short
5 and variable time of duration of exposure increases the possibility of differences between the *in*
6 *vivo* data resulting from differences in tumor progression as well as a decreased ability by the
7 shortened time of observation for full expression of the tumor response.
8
4.3. Co-exposures of Haloacetates and Other Solvents
10
11   As noted by Caldwell et al. (2008b), drinking water exposure data suggest co-exposure of
12 TCE and its haloacetic acid metabolites, TCA and DCA, is not an uncommon event as DCA and
13 TCA are the two most abundant haloacetates in most water supplies (Weisel et al., 1999,
14 Boorman et al., 1999). Dibromoacetic acid (DBA) concentrations have also been reported to
15 range up to approximately 20 μg/L in finished water and distribution systems (Weinberg et al.,
16 2002). Caldwell et al. (2008b) have also noted that co-exposure in different media also occurs
17 with solvents like perchloroethylene (PERC) that may share some MOAs, targets of toxicity, and
18 common metabolites that can therefore potentially affect TCE health risk (Wu and Schaum,
19 2000). Some of the information contain in the following sections have been excerpted from the
20 discussions by Caldwell et al (2007b) regarding the implications for the risk of TCE exposure as
21 modulated by co-exposures to haloacetates and other solvents that have been studied and reported
22 in the literature.
23
4.3.1. Carbon tetrachloride, DCA, TCA: Implications for MOA from Co-exposures
25
26   Studies of specific combinations of TCE and chemicals co-located in contaminated areas
27 have been reported by Caldwell et al. (2008b). For carbon tetrachloride "Pretreatment with TCE
28 in drinking water at levels as low as 15 mM for three days has been reported to increase
29 susceptibility to liver damage to subsequent exposure to a single IP injection of 1 mM/kg carbon
30 tetrachloride (CCl_4) in Fischer 344 rats [Steup et al., 1991]. Potential mechanistic explanations
31 for this observation included altered metabolism, decreased hepatic repair capability, decreased
32 detoxification ability, or combination of one or more of the above activities. Simultaneous
33 administration of an oral dose of TCE (0.5ml/kg) has also been reported to increase the liver
34 injury induced by an oral dose of 0.05 ml/kg CCl_4 [Steup et al., 1993].   The authors suggested
35 that TCE appeared to impair the regenerative activity in the liver, thus leading to increased
36 damage when CCl_4 is given in combination with TCE."
37
38   As discussed above in Section 4.2, initiation studies are in themselves a co-exposure. The
39 study of Bull et al. (2004) is included here as it not only used a co-exposure of vinyl carbamate
40 with TCE metabolites, but also used carbon tetrachloride as a co-exposure as well. The rationale
41 for this approach was that co-exposure of TCE (and therefore to its metabolites) and CCl_4 are
42 likely to occur as they are commonly found together at contaminated sites. Bull et al. (2004)
43 hypothesized that modification of tumor growth rates is an indication of promotion rather than

1 effects on tumor number, and that by studying tumor growth rates they could classify carcinogens
2 by their MOAs. B6C3F1 male mice were initiated with vinyl carbamate (3 mg/kg) at 2 weeks of
3 age and then treated with DCA, TCA, CCl_4, (0.1, 0.5, or 2.0 g/l for DCA and TCA; 50, 100 or
4 500 mg/kg CCL$_4$ in 5% Alkamuls via gavage) in pair-wise combinations of the three for 18 to 36
5 weeks. The exposure level of CCL$_4$ to 5, 20 and 50 mg/kg was reported to be reduced at week 24
6 due to toxicity for CCl_4.   The number of mice in each group was reported to be 10 with the study
7 divided into 5 segments. There were evidently differences between treatment segments as the
8 authors state that " because of some significant quantitative differences in results that were
9 obtained with replicate experiments treated in different time frames, the simultaneous controls
10 have been used in the analysis and presentation of these data." As with Bull et al. (2002), the
11 interpretation of the results of the study is limited by a low number of animals per group, short
12 duration time of exposure and limited examination and reporting of results. For example, a
13 sample of 100 out of the 8000 lesions identified in the study was examined to verify that the
14 general descriptor of neoplastic and non-neoplastic lesion was correctly labeled with "tumors"
15 describing a combination of hyperplastic nodules, adenomas, and carcinomas. No incidence data
16 was reported by the authors, but general lesion growth information included mean lesion volume
17 and multiplicity of lesions (numbers of lesions/mouse). Using these reported indices, there
18 appeared to be differences in treatment-related effects.
19
20   Each treatment was examined alone and then in differing combinations with each other.
21 Mice initiated with vinyl-carbamate, but without further exposure to the other toxicants, were
22 reported to have a few lesions that were of small size during the examination period (20-36
23 weeks). At 30 weeks of CCl_4 exposure, there was a dose-related response reported for
24 multiplicity but mean lesion size was smaller at the highest dose in initiated animals. At 36
25 weeks, DCA exposure was reported to increase multiplicity at the two highest exposure levels
26 and increased lesion size at all levels compared to initiated-only animals. However, at a similar
27 level of induction, multiplicity and mean size of those lesions resulting from DCA treatment were
28 reported to be much smaller in comparison with CCl_4 treatment (i.e., a 20-fold difference for
29 lesion volume). At 36 weeks, treatments with the same concentration of TCA or DCA induced
30 similar multiplicity, but the mean lesion volume was reported to be approximately 4-fold greater
31 in tumors induced by DCA as compared to TCA, and in animals treated with DCA multiplicity
32 had reached a plateau by 24 weeks rather than 36 for those treated with TCA. Thus, using
33 multiplicity of lesions and lesion volume as indicators of differences in MOA, exposure to CCl_4,
34 DCA, and TCA appeared to produce distinct differences in results in animals previously treated
35 with vinyl carbamate.
36
37   Simultaneous co-exposure of differing combinations of CCl_4, DCA, and TCA were
38 reported to give more complex results between 24 and 36 weeks of observation but to show that
39 co-exposure had effects on lesion multiplicity and volume in initiated animals. At 36 weeks,
40 TCA co-exposure appeared to reduce the lesion volume of either DCA- or CCl_4-induced lesions
41 after vinyl carbamate treatment. Similarly, DCA co-exposure was reported to reduce the lesion
42 volume of either TCA- or CCl_4-induced lesions when each was given alone after vinyl carbamate
43 treatment. With regard to multiplicity, TCA co-exposure was reported to reduce DCA-induced

1   multiplicity only at the lowest dose of TCA while co-exposure with DCA increased multiplicity
2   of CCl_4-induced lesions at all exposure levels. At 24 weeks, there appeared to be little effect on
3   mean lesion volume by any of the co-exposures but DCA co-exposure decreased multiplicity of
4   TCA-induced lesions (up to 3-fold) while TCA treatment slightly increased the number of CCl_4-
5   induced multiplicity (1.6-fold). This study confirms that short duration of exposure to all three of
6   these chemicals can cause lesions in already exposed to vinyl carbamate, and suggests that
7   combinations of these agents differentially influence lesion number and growth rates. The
8   authors have interpreted their results to indicate differences in MOA between such treatments.
9   However, the limitations of the study limit conclusions regarding how such co-exposure may be
10   able to affect toxicity and tumor induction and what the MOA is for each of these agents. This is
11   especially true at lower and more environmentally relevant concentrations given for longer
12   durations to uninitiated animals (Caldwell et al 2007b).
13
14   **4.3.2. Chloroform, DCA, and TCA Co-exposures: Changes in Methylation Status**
15
16   In Section 3.4.2.2, information on the effects of TCE and its metabolites was presented in
17   regard to effects on methylation status. After 7 days of gavage dosing, TCE, TCA and DCA
18   were reported to increased hypomethylation of the promoter regions of c-Jun and c-Myc genes in
19   mouse whole liver DNA, however, Caldwell and Keshava (2006) concluded that
20   hypomethylation did not appear to be a chemical-specific effect at the concentration used. Bull
21   et al. (2004) suggested that hypomethylation occurs at higher exposure levels than those that
22   induce liver tumors for TCE and its metabolites.   Along with studies of methylation changes
23   induced by a exposure to a single agent a Pereira et al (2001) have attempted to examine the
24   effects on methylation changes from co-exposures. This study was also reviewed by Caldwell et
25   al (2007b).
26
27   Pereira et al. (2001) hypothesized that changes in the methylation status of DNA can be a
28   key event for MOA for DCA- and TCA- induced liver carcinogenicity through changes in gene
29   regulation, and that chloroform ($CHCl_3$) co-exposure may result in modification of DNA
30   methylation. After 17 days of exposure of exposure to $CHCl_3$ (0, 400, 800, 1600 mg/l, n= 6 mice
31   per treatment group) female B6C3F1 mice were co-exposed to DCA or TCA (500 mg/kg) during
32   the last 5 days of exposure to chloroform. As noted by Caldwell et al (2007b), Pereira et al.
33   (2001) reported the effects of hypomethylation of the promoter region of *c-Myc* gene and on
34   expression of its mRNA in the whole livers of female B6C3F1 mice and thus, these results
35   represent composite changes in DNA methylation status from a variety of cell types and for
36   hepatocytes lumped from differing parts of the liver lobule. When given alone, DCA, TCA, and
37   to a lesser extent, the highest concentration of $CHCl_3$ (1600 mg/L), was reported to decrease
38   methylation of the *c-myc* promoter region. Co-administration of $CHCl_3$ (at 800 and 1600 mg/l)
39   was reported to decrease DCA-induced hypomethylation while exposure to $CHCl_3$ had no effect
40   on TCA-induced hypomethylation. Treatment with DCA, TCA, and, to a lesser extent $CHCl_3$,
41   was reported to increase levels of *c-myc* mRNA. While expression of *c-myc* mRNA was
42   increased by DCA or TCA treatment, increasing co-exposures to $CHCl_3$ were reported to
43   attenuate the actions of DCA but not TCA. Thus, differences in methylation status and

1 expression of the *c-myc* gene induced by DCA or TCA exposure was reported to be differentially
2 modulated by co-exposure to $CHCl_3$. The authors suggest these differences support differing
3 actions by DCA and TCA. However, whether these changes represent key events in the
4 induction of liver cancer is a matter of debate, especially as a "snapshot in time" approach for
5 such a non-specific endpoint (Caldwell et al 2007b).
6
7   In a co-exposure study in which an "initiating agent" was used as a co-exposure along
8 with other co-exposure, Pereira et al. (2001) treated male and female 15-day old B6C3F1 mice
9 with N-methyl-N-nitrosourea (MNU) (a cause of liver and kidney tumors) and then, starting at 5
10 weeks of age, treated them with DCA (3.2 g/L) or TCA (4.0 g/L) along with co-exposure to
11 $CHCl_3$ (0, 800 or 1600 mg/l) for 36 weeks. Mice were reported to be examined for evidence of
12 promotion of liver and kidney tumors. The numbers of animals in the exposure groups were
13 highly variable, ranging from 25 (female initiated mice exposed to DCA) to 6 (female initiated
14 mice exposed to DCA and 1600 mg/L $CHCl_3$), thus limiting the power of the study to ascertain
15 treatment-related changes. However, unlike Bull et al (2004), all liver tissues were examined
16 with incidences of foci, adenomas, carcinomas, and both adenoma and carcinoma reported
17 separately for treatment groups. Multiplicity for a combination of adenomas and carcinomas
18 were reported as well as the tincture of foci and tumors.
19
20   Although as noted by Caldwell et al (2007b), the statistical power of the study to detect
21 change was very low, an examination of the pattern of tumors induced by co-exposure to MNU
22 and TCE metabolites in female mice suggested that: (1) DCA exposure increased the incidence
23 of adenomas but not carcinomas; (2) TCA increased incidence of carcinomas with little change in
24 adenoma incidence; (3) co-exposure to 800 and 1600 mg/l of $CHCl_3$ decreased adenoma
25 incidence by DCA treatment but not TCA; and (4) $CHCl_3$ co-exposure decreased multiplicity of
26 TCA-induced tumors and foci, but not for DCA. Caldwell et al (2007b) also note that this study
27 suggests a gender-related effect on tumor induction from this study with; (1) adenoma incidences
28 similar in male and female mice treated with DCA, but carcinoma incidence greater in males; (2)
29 adenoma and carcinoma incidences greater in males than females treated with TCA; (3) tumor
30 multiplicity similar in both genders for DCA treatments, but lower in females mice for TCA; and
31 (4) less of an inhibitory effect by $CHCl_3$ on adenoma incidence from DCA exposure in male
32 mice.
33
34   Pereira et al. (2001) also described the tinctural characteristics of the specific lesions
35 induced by their co-exposure treatments. Both foci and tumors induced by DCA exposure in
36 "initiated" mice were reported to be over 95% eosinophilic in females, while in males, 89% of
37 the foci were eosinophilic and 91% of tumors were basophilic. Thus, not only was there a
38 gender-related difference in the incidences of tumors and foci but also foci and tumor phenotype.
39 $CHCl_3$ co-exposure was reported to change the DCA-induced foci from primarily eosinophilic to
40 basophilic (i.e., 11% vs. 75% basophilic) in male mice co-exposed to MNU. In male and female
41 mice, TCA-induced tumors and foci were basophilic with no effect of $CHCl_3$ on phenotype in
42 MNU treated mice.
43

4.3.3. Co-exposures to Brominated Haloacetates: Implications for Common MOAs and Background Additivity to Toxicity.

As noted by Caldwell et al (2007b), along with chlorinated haloacetates and other solvents, co-exposures with TCE and brominated haloacetates may occur through drinking water. These compounds may affect TCE toxicity in a similar fashion to their chlorinated counterparts. As bromide concentrations increase, brominated haloacetates increase in the water supply.

Kato-Weinstein et al. (2001) administered dibromoacetate (DBA), bromochloroacetate (BCA), bromodichloroacetate (BDCA), TCA, and DCA in drinking water at concentrations of 0.2–3 g/L for 12 weeks to B6C3F1 male mice. The focus of the study was to determine the similarity in action between the brominated and chlorinated haloacetates. Each of the haloacetates, given individually, were reported to increase liver/body weight ratios in a dose-dependent manner. The dihaloactates, DCA, BCA and DBA, caused liver glycogen accumulation both by chemical measurements in liver homogenates and in ethanol-fixed liver sections (to preserved glycogen) stained with PAS. For DCA, a maximal level of glycogen increase was observed at 4 weeks of exposure at a 2 g/l exposure concentration. They report a 1.60-fold of control % liver/body weight and 1.50-fold of control glycogen content after 8 weeks of exposure to 2 g/l DCA in male B6C3F1 mice. The baseline level of glycogen content (~ 60 mg/g) and the increase in glycogen after DCA exposure was consistent with the results reported by Pereira et al. (2004). The % liver/body weight data for control mice was for animals sacrifice at 20 weeks of age. The 4-12 week exposure to DCA were staggered so that all animals would be 20 weeks of age at sacrifice. Thus, the animals were at differing ages at the beginning of DCA treatments between the groups. However, as with Pereira et al. (2004) the ~ 10% increase in liver mass that the glycogen increases represent are lower than the total increase in liver mass reported for DCA exposure. The authors noted possible contamination of BCA with small percentages of DCA and DBA in their studies (i.e., 84% BCA, 6% DCA and 8% DBA). The trihaloacetates (TCA and low concentrations of BDCA) were reported to produce slight decreases in liver glycogen content, especially in the central lobular region in cells that tended to accumulate glycogen in control animals. These effects on liver glycogen were reported at the lowest dose examined (i.e., 0.3 g/l). At the highest concentration, BDCA was reported to induce a pattern of glycogen distribution similar to that of DCA in mice.

All dihaloacetates were reported to reduce serum insulin levels at high concentrations. Conversely, trihaloacetates were reported to have no significant effects on serum insulin levels. For the study of peroxisome proliferation and DNA synthesis, mice were treated to BCA, DBA and BDCA for 2, 4, or 26 weeks. The effects on DNA synthesis were small for all brominated haloacetates with only DBA reported to show a significant increase in DNA synthesis at 2 and 4 weeks but not at 26 weeks (increase in DNA synthesis was 3-fold of the highest control level). Of note is the highly variable level of DNA synthesis reported for control values that varied to a much higher degree (~3- 6-fold variation within control groups at the same time points) than did treatment-related changes. DBA was the only brominated haloacetate that was reported to consistently increased PCO activity as a percentage of control values (actual values and

1　variability between controls were not reported) with a 2-3 fold increase in PCO activity at 0.3 to
2　3.0 g/l DBA. DBA-induced PCO activity increases were reported to be limited to 2-4 weeks of
3　treatment in contrast to TCA, which the authors reported to increase PCO activity consistently
4　over time.
5
6　　　Tao et al. (2004) reported DNA methylation, glycogen accumulation and peroxisome
7　proliferation after exposure of female B6C3F1 mice and male Fischer 344 rats exposed to 1 or 2
8　g/l DBA in drinking water for 2 to 28 days. DBA was reported to induce dose-dependent DNA
9　hypomethylation in whole mouse and rat liver after 7 days of exposure with suppression
10　sustained for the 28-day exposure period. The expression of mRNA for *c-Myc* in mice and rats
11　and mRNA expression of the *IGF-II* gene in female mice were reported to be increased during
12　the same period. Both rats and mice were reported to exhibit increased glycogen with mice
13　having increased levels at 2 day and rats at 4 days. DBA was reported to cause an increase in
14　lauroyl-CoA oxidase activity (a marker of peroxisome proliferation) in both mice (after 7 days)
15　and rats (after 4 days) that was sustained for 28 days. Methylation changes reported here for
16　DBA exposure in rats and mice are consistent with those reported for TCA and DCA by Pereira
17　et al. (2001) in mice. The pattern of glycogen accumulation was also similar to that reported for
18　DCA by Kato-Weinstein et al. (2001) and suggests that the brominated analogues of TCE
19　metabolites exhibited similar actions as their chlorinated counterparts. In regard to peroxisomal
20　enzyme activities Kato-Weinstein et al. (2001) reported PCO activity to be limited to 2-4 weeks
21　with Tao et al (2004) reporting lauroyl-CoA oxidase activity to be sustained for the lengths of the
22　study (28-days) for DBA.
23
24　　　As noted by Caldwell et al (2007b), given the similarity of DCA and DBA effects, it is
25　plausible that DBA exposure also induces liver cancer. Melnick et al. (2007) reported the results
26　of DBA exposure to F344/N rats and B6C3F1 mice exposed to DBA for 3 months or 2 years in
27　drinking water (0, 0.05, 0.5, or 1.0 g/l DBA for 2 years). Neoplasms at multiple sites were
28　reported in both species exposed to DBA for 2 years with no effects on survival and little effect
29　on mean body weight in either species.   Similar to TCE, DCA and TCA, the liver was reported
30　to be a target of DBA exposure. After 3-months of exposure, there were dose-related increases in
31　hepatocellular vacuolization and liver weight reported in rats and mice described as "glycogen-
32　like."   The authors report that the major neoplastic effect of DBA in rats was induction of
33　malignant mesotheliomas in males and increased incidence of mononuclear cell leukemia in
34　males and females. For mice, the major neoplastic effect of DBA exposure was reported to be
35　the increased incidence of hepatocellular adenomas and carcinomas at all exposure levels.   In
36　addition to these liver tumors, hepatoblastomas were also reported to be increased in all exposure
37　groups of male mice and exceeded historical control rates. The incidence of alveolar/bronchiolar
38　adenoma and carcinoma was reported to be increased in the 0.5 g/L group of male mice along
39　with marginal increases in alveolar hyperplasia in DBA-treated groups. The authors reported that
40　the increases in hepatocellular neoplasms were not associated with hepatocellular necrosis or
41　regenerative hyperplasia and concluded that an early increase in hepatocyte proliferation were
42　not likely involved in the MOA for DBA because no increases in hepatocyte DNA labeling index

1   were observed in mice exposed for 26 days and the slight increase that occurred in male F344
2   rats was not accompanied by an increase in liver tumor response.
3
4   As noted by Caldwell et al (2007), the results of Kato-Weinstein et al. (2001), Tao et al.
5   (2004), and Melnick et al. (2007) are generally consistent for DBA and show a number of
6   activities that may be common to TCE metabolites (i.e., brominated and chlorinated haloacetate
7   analogues generally have similar effects on liver glycogen accumulation, serum insulin levels,
8   peroxisome proliferation, hepatocyte DNA synthesis, DNA methylation status, and
9   hepatocarcinogenicity).   It is therefore plausible that these effects may be additive in situations
10   of co-exposure. However, as noted by Melnick et al (2007), methylation status, events associated
11   with PPARα agonism, hepatocellular necrosis, and regenerative hyperplasia are not established
12   as key events in the MOA of these agents, and the MOAs for DCA- and DBA- induced liver
13   tumors are unknown.
14
15   **4.3.4. Co-exposures to Ethanol: Common Targets and MOAs**
16
17   As noted in the U.S. Environmental Protection Agency's draft TCE assessment [U.S.
18   EPA, 2001], alcohol consumption is a common co-exposure that has been noted to affect TCE
19   toxicity with TCE exposure cited as potentially increasing the toxicity of methanol and ethanol,
20   not only by altering their metabolism to aldehydes, but also by altering their detoxification (e.g.,
21   similar to the "alcohol flush" reported for those who have an inactive aldehyde dehydrogenase
22   allele). As noted by Caldwell et al (2007b) "chemical co-exposures from both the environment
23   and behaviors such as alcohol consumption may have effects that overlap with TCE in terms of
24   active agents, pharmacokinetics, pharmacodynamics, and/or target tissue toxicity." Caldwell et
25   al (2007b) also note:
26
27   In their review of solvent risk (including TCE), Brautbar and Williams (2002) suggest
28   that laboratory testing that is commonly used by clinicians to detect liver toxicity may not
29   be sensitive enough to detect early liver hepatotoxicity from industrial solvents and that
30   the final clinical assessment of hepatotoxicity and industrial solvents must take into
31   account synergism with medications, drugs of use and abuse, alcohol, age-dependent
32   toxicity, and nutrition. Although many of these factors may be important, the focus in
33   this section is on the effects of ethanol. Contemporary literature reports effects similar to
34   those of TCE's and previous reports indicate ethanol consumption impacts TCE toxicity
35   in humans, affects the pharmacokinetics and toxicity of TCE in rats, and is also a risk
36   factor for cancer.
37
38   The association between malignant tumors of the upper gastrointestinal tract and liver and
39   ethanol consumption is based largely on epidemiological evidence, and thought to be
40   causally related [Bradford et al., 2005; Badger et al., 2003]. Studies of the mechanisms of
41   ethanol carcinogenicity have suggested the importance of its metabolism, including
42   induction of CYP2E1 associated increases in production of reactive oxygen species and
43   enhanced activation of a variety of pro-carcinogens, alteration of retinol and retinoic acid

metabolism, and the actions of the metabolite acetaldehyde [Badger et al., 2003]. While ethanol is primarily metabolized by alcohol dehydrogenase, it undergoes simultaneous oxidation to acetate by hepatic P450s, primarily CYP2E1. Both chronic ethanol consumption as well as TCE treatment induces CYP2E1 [U.S. EPA, 2001]. Oneta et al. (2002) report that even at moderate chronic ethanol consumption, hepatic CYP2E1 is induced in humans, which they suggest, may be of importance in the pathogenesis of alcoholic liver disease; of ethanol, drug, and vitamin A interactions; and in alcohol-associated carcinogenesis. Induction of CYP2E1 can cause oxidative stress to the liver from nicotinamide dinucleotide phosphate (NADPH)-dependent reduction of dioxygen to reactive products even in the absence of substrate, and subsequent apoptosis [Badger et al., 2003]. Bradford et al. (2005) suggest that CYP2E1, and not NADPH oxidase, is required for ethanol-induced oxidative DNA damage to rodent liver but that NADPH oxidase-derived oxidants are critical for the development of ethanol-induced liver injury.

There is increasing evidence that acetaldehyde, which is toxic, mutagenic, and carcinogenic, rather than alcohol is responsible for its carcinogenicity [Badger et al., 2003]. Mitochondrial aldehyde dehydrogenase (ALDH2) disposes of acetaldehyde generated by the oxidation of ethanol, and ALDH2 inactivity through mutation or polymorphism has been linked to esophageal cancer in humans (everyday drinkers and alcoholics) [Badger et al., 2003]. For instance, increased esophageal cancer risk was reported for patients with the ALDH3*1 polymorphism as well as increased acetaldehyde in their saliva. TCE exposure has also been reported to induce a similar alcohol flush in humans which may be linked to its ability to decrease ALDH activities at relatively low concentrations and thus conferring a similar status to individuals with inactive ALDH2 allele [Wang et al., 1999]. Whether the MOA for the buildup of acetaldehyde after ethanol and TCE co-exposure is: (1) the induction of CYP2E1 by TCE resulting in increased metabolism to acetaldehyde; (2) inhibition of ALDH and thus reduced clearance of acetaldehyde, or (3) a number of other actions are unknown. Crabb et al. (2001) reported 20-30% reductions in ALDH2 protein level by PPARα agonists (Clofibrate treatment in rats and WY treatment in both wild and PPARα-null mice). This could be another pathway for TCE-induced effects on ethanol metabolism. It is an intriguing possibility that the reported association between the increased risk of human esophageal cancer and TCE exposure [Scott and Chiu, 2006] could be related to TCE effects on mitochondrial ALDH, given a similar association of this endpoint with ethanol consumption or ALDH polymorphism.

Finally, ethanol ingestion may have significant effects on TCE pharmacokinetics. Baraona et al. (2002 a,b) reported that chronic, but not acute, ethanol administration increased the hepatotoxicity of peroxynitrite, a potent oxidant and nitrating agent, by enhancing concomitant production of nitric oxide and superoxide. They also reported that nitric oxide mediated the stimulatory effects of ethanol on blood flow. Ethanol markedly enhanced portal blood flow (2-fold increase), with no changes in the hepatic, splenic, or pancreatic arterial blood flows in rats.

1 **4.3.5. Co-exposure Effects on Pharmacokinetics: Predictions Using Physiologically Based**
2 **Pharmacokinetic (PBPK) Models**
3
4 Along with experimental evidence that has focused on chronic and acute experiments
5 using rodents, the potential pharmacokinetic modulation of risk has also been recently published
6 reports using physiologically based pharmacokinetic (PBPK) models that may be useful in
7 predicting co-exposure effects. Caldwell et al. (2008b) also examined and discussed these
8 approaches and note:.
9
10 An important issue for prediction of the effects and relationship on MOAs by co-exposure
11 is the degree to which modulation of TCE toxicity by other agents can be quantified.
12 Pharmacokinetics or the absorption, distribution, metabolism, and elimination of an agent,
13 can be affected by internal and external co-exposure. Such information can help to
14 identify the chemical species that may be causally associated with observed toxic
15 responses, the MOA, and ultimately identify potentially sensitive subpopulations for an
16 effect such as carcinogenicity.
17
18 Physiologically based pharmacokinetic (PBPK) models are often used to estimate and
19 predict the toxicologically relevant dose of foreign compounds in the body and have been
20 developed to predict effects on pharmacokinetics that are additive or less or greater than
21 additive. One of the first such models was developed for TCE [Andersen et al., 1987].
22 Given that TCE, PERC, and methyl chloroform (MC) are often found together in
23 contaminated groundwater, Dobrev et al. (2001) attempted to investigate the
24 pharmacokinetic interactions among the three solvents to calculate defined "interaction
25 thresholds" for effects on metabolism and expected toxicity. Their null hypothesis was
26 defined as competitive metabolic inhibition being the predominant result for TCE given
27 in combination with other solvents. Gas uptake inhalation studies were used to test
28 different inhibition mechanisms. A PBPK model was developed using the gas uptake
29 data to test multiple mechanisms of inhibitory interactions (i.e., competitive,
30 noncompetitive, or uncompetitive) with the authors reporting competitive inhibition of
31 TCE metabolism by MC and PERC in simulations of pharmacokinetics in the rat.
32 Occupational exposures to chemical mixtures of the three solvents within their Threshold
33 Limit Value (TLV)/TWA limits were predicted to result in a significant increase (22%) in
34 TCE blood levels compared with single exposures.
35
36 Dobrev et al. (2002) extended this work to humans by developing an interactive human
37 PBPK model to explore the general pharmacokinetic profile of two common biomarkers
38 of exposure, peak TCE blood levels, and total amount of TCE metabolites generated in
39 rats and humans. Increases in the TCE blood levels were predicted to lead to higher
40 availability of the parent compound for GSH conjugation, a metabolic pathway that may
41 be associated with kidney toxicity/carcinogenicity. A fractional change in TCE blood
42 concentration of 15% for combined TLV exposures of the three chemicals (25/50/350
43 ppm of PERC/TCE/MC) resulted in a predicted 27% increase of the S-(1, 2-

1   dichlorovinyl)-L-cysteine (DCVC) metabolites, indicating a nonlinear risk increase due to
2   combined exposures to TCE. Binary combinations of the solvents produced GST-
3   mediated metabolite levels almost twice as high as the expected rates of increase in peak
4   blood levels of the parent compound. The authors suggested that using parent compound
5   peak blood levels (a less sensitive biomarker) would result in two to three times higher
6   (i.e., less conservative) estimates of potentially safe exposure levels. In regard to the
7   detection of metabolic inhibition by PERC and MC, the simulations showed TCE blood
8   concentrations to be the more sensitive dose metric in rats, but the total of TCE
9   metabolites to be the more sensitive dose measure in humans. Finally, interaction
10 thresholds were predicted to be occurring at lower levels in humans than rats.

12 Thrall and Poet (2000) investigated the pharmacokinetic impact of low-dose co-exposures
13 to toluene and TCE in male F344 rats *in vivo* using a real-time breath analysis system
14 coupled with PBPK modeling. The authors report that, using the binary mixture to
15 compare the measured exhaled breath levels from high- and low-dose exposures with the
16 predicted levels under various metabolic interaction simulations (competitive,
17 noncompetitive, or uncompetitive inhibition), the optimized competitive metabolic
18 interaction description yielded an interaction parameter Ki value closest to the Michaelis-
19 Menten affinity parameter (K_M) of the inhibitor solvent. This result suggested that
20 competitive inhibition is the most plausible type of metabolic interaction between these
21 two solvents.

23 Isaacs et al. (2004) have reported gas uptake co-exposure data for $CHCl_3$ and TCE. The
24 question as to whether it is possible to use inhalation data in combination with PBPK
25 modeling to distinguish between different metabolic interactions was addressed using
26 sensitivity analysis theory. Recommendations were made for design of optimal
27 experiments aimed at determining the type of inhibition mechanisms resulting from a
28 binary co-exposure protocol. This paper also examined the dual nature of inhibition of
29 each chemical in the pair to each other, which is that TCE and $CHCl_3$ were predicted to
30 interact in a competitive manner. Even though as stated by Dobrev et al. (2001), other
31 solvents inhibit TCE metabolism, it is also possible to quantify the synergistic interaction
32 that TCE has on other solvents, using techniques such as gas uptake inhalation exposures.

34 Haddad et al. (2000) has developed a theoretical approach to predict the maximum impact
35 that a mixture consisting of co-exposure to dichloromethane, benzene, TCE, toluene,
36 PERC, ethylbenzene, m-, p-, and o-xylene, and styrene would have on venous blood
37 concentration due to metabolic interactions in Sprague-Dawley rats. Two sets of
38 experimental co-exposures were conducted. The first study evaluated the change in
39 venous blood concentration after a 4 hour constant inhalation exposure to the 10 chemical
40 mixtures. This experiment was designed to examine metabolic inhibition for this
41 complex mixture. The second study was designed to study the impact of possible enzyme
42 induction by using the same inhalation co-exposure after a 3 day pretreatment with the
43 same 10 chemical mixture. The resulting venous concentration measurements for TCE

from the first study were consistent with metabolic inhibition theory. The 10-chemical
mixture was the most complex co-exposure used in this study. The authors stated that as
mixture complexity increased, the resulting parent compound concentration time courses
changed less, an observation which is consistent with metabolic inhibition. For the
pretreatment study, the authors found a systematic decrease in venous concentration (due
to higher metabolic clearance) for all chemicals except PERC. Overall, these studies
suggest a complex metabolic interaction between TCE and other solvents.

A PBPK model for TCE including all its metabolites and their interactions can be
considered a mixtures model where all metabolites have a common starting point in the
liver. An integrated approach taking into account TCE metabolites and their metabolic
inhibition and interactions among each other is suggested in Chiu et al. (2006b).

5. Potentially Susceptible Life Stages and Conditions That May Alter Risk of Liver Toxicity and Cancer

As described in Sections 1.2., 3.2.2., 3.2.6, 4.2.1, 4.2.2, 4.2.3., and 4.2.4, there are a
number of conditions that are associated with increased risk of liver cancer and toxicity that
include age, use of a number of prescription medications including fibrates and statins, disease
state (e.g., diabetes, NALD, viral infections) and exposure to external environmental
contaminants that have an affect on TCE toxicity and targets. Obviously epigenetic and genetic
factors play a role in determining the risk to the individual. In terms of liver cancer, there is
general consensus that despite the associations that have been made with etiological factors and
the risk of liver cancer, the mechanism is still unknown. The MOA of TCE toxicity is also
unknown but exposure to TCE and its metabolites have shown in rodent models to induce liver
cancer and in a fashion that is not consistent with only a hypothesized MOA of PPARα receptor
activation that is in need of revision. However, multiple TCE metabolites have been shown to
also induce liver cancer with varying effects on the liver that have also been associated with early
stages of neoplasia (glycogen storage) or other actions associated with risk of
hepatocarcinogenicity. The growing epidemic of obesity has been suggested to increase the risk
of liver cancer and may reasonably increase the target population for TCE effects on the liver.

Lifestyle factors such as ethanol ingestion have not only been shown to increase liver
cancer risk in those who already have fatty liver, but also to increase the toxicity of TCE.
However, as noted by Caldwell et al (2007b), while there is evidence to suggest that TCE
exposure may increase the risk of liver toxicity and cancer, there are not data to support a
quantitative estimate of how co-exposures may modulate that risk.

These findings can also serve to alert the risk manager to the possibility that multiple
internal and external exposures to TCE that may act via differing MOAs for the
production of liver effects. This information suggests a possible lack of "zero"
background exposures and can help identify potential susceptible populations.

1   Background levels of haloacetates in drinking water may add to the cumulative exposure
2   an individual receives via the metabolism of TCE. The brominated haloacetates
3   apparently share some common effects and pathways with their chlorinated counterparts.
4   Thus, concurrent exposure of TCE, its metabolites, and other haloacetates may pose an
5   additive response as well as an additive dose. However, personal exposures are difficult
6   to ascertain and the effects of such co-exposures on toxicity are hard to quantify. EPA's
7   guidance on cumulative risk assessments directs "each office to take into account
8   cumulative risk issues in scoping and planning major risk assessments and to consider a
9   broader scope that integrates multiple sources, effects, pathways, stressors, and
10   populations for cumulative risk analyses in all cases for which relevant data are available"
11   [U.S. EPA, 1997]. Widespread exposure to possible background levels of TCE
12   metabolites or co-contaminants and other extrinsic factors have the potential to affect
13   TCE toxicity. However, the available data for co-exposures on TCE toxicity appears
14   inadequate for quantifying these effects, particularly at environmental levels of
15   contamination and exposure. Thus, the risk manager and assessor are going to be limited
16   by not having information regarding either (1) the type of exposure data necessary to
17   assess the magnitude of co-exposures that may affect toxicity, or (2) the potential
18   quantitative impacts of these co-exposures that would enable specific adjustments to risk.
19   Nonetheless, the risk manager should be aware that qualitatively a case can be made that
20   extrinsic factors may affect TCE toxicity.
21

22   **6.   Uncertainty and Variability**
23

24   Along with general conclusions about the coherence of data that enable conclusions about
25 effects on the liver shown through experimental studies of TCE, there have also been extensive
26 discussions throughout this report regarding the specific limitations of experimental studies
27 whose design was limited by small and varying groups of animals and variability in control
28 responses as well as reporting deficiencies. Section 3.2.5. has brought forward the uncertainty in
29 the MOA for liver cancer in general. The consistency of different animal models with human
30 HCC is described in Section 3.3., with Section 3.2.2. providing a discussion of the promise and
31 limitations of emerging technologies to study the MOAs of liver can in general and for TCE
32 specifically. Issues regarding the target cell for HCC and the complexities of studying the MOA
33 for a heterogeneous disease are described in Sections 3.2.4. and 3.2.8., respectively. Finally, the
34 uncertainty regarding key events in how activation of the PPARα receptor my lead to
35 hepatocarcinogenesis and the problems with extrapolation of results using the common paradigm
36 to study them (exposure to high levels of WY-14,643 in abbreviated bioassays in knockout mice)
37 are outlined in Section 3.5.1. As such uncertainties are identified future research can focus on
38 resolving them.

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