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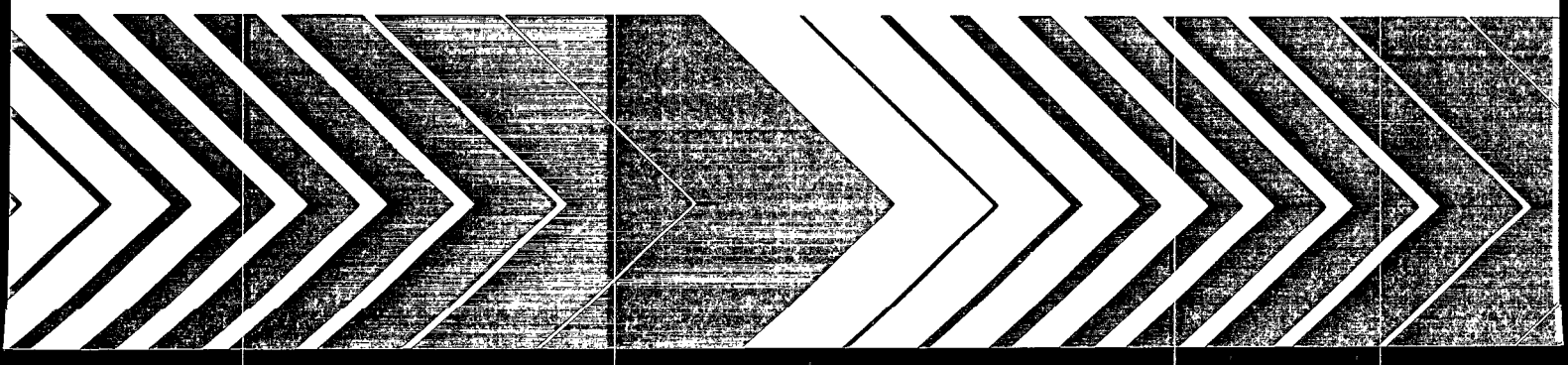
Technical Support Document on Risk Assessment of Chemical Mixtures



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Technical Support Document on Risk Assessment of Chemical Mixtures

Environmental Criteria and Assessment Office
Office of Health and Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

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PREFACE

The preparation of the mixtures Technical Support Document (TSD) was recommended in 1985 by the U.S. EPA Science Advisory Board (SAB) panel that reviewed the Agency's mixtures guidelines. Following completion of the external review draft in December, 1987, the TSD was reviewed by both Agency and external experts in the field of chemical mixtures risk assessment. Among the external reviewers were Ron Wyzga (EPRI), who was a member of the original SAB review panel for the mixtures guidelines, and Richard Cothern, who is currently a member of the SAB.

Unique sections of the TSD include: an overview of available toxicity data on complex mixtures and binary exposures (ch. 2) and mechanisms of interaction (ch. 3), an estimate of the maximum synergistic effect observed for environmental chemicals (ch. 2), an evaluation of quantitative methods (statistics and models) that have been used in characterizing interactions (ch. 4), a summary of the U.S. EPA's interaction data base (appendix A), recommendations for revisions to the existing mixtures guidelines (ch. 5) and recommendations for future research relevant to risk assessment (ch. 6). The two most significant conclusions in this document are 1) that the available literature is extremely inadequate for use in quantifying the extent of synergism expected from environmental exposures, and 2) that validation of *in vitro* and short-term *in vivo* studies seems to offer the most promise for improving risk assessments of complex mixtures.

The first draft of this document was prepared by Syracuse Research Corporation under contract no. 68-C8-0004 with chapters contributed by the Department of Environmental Health of the University of Cincinnati under cooperative agreement no. CR-813569-01-0, and by staff of the Agency's Environmental Criteria and Assessment Office in Cincinnati. The literature search perfomed is current as of August, 1988.

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1. INTRODUCTION AND BACKGROUND

This technical support document is a supplement to the U.S. Environmental Protection Agency's Guidelines for the Health Risk Assessment of Chemical Mixtures published on September 24, 1986 (U.S. EPA, 1986a, 1987a). This document was developed in response to a recommendation of the Science Advisory Board (SAB). It discusses available toxicity and interaction information useful in assessing human health risks from mixtures. In addition, applicable mathematical models and statistical techniques are reviewed and research needs are identified. The results of the above information are discussed along with implications for the current guidelines.

1.1. THE CHEMICAL MIXTURE GUIDELINES

The mixtures guidelines are intended to guide Agency analysis of information relating to health effects data on chemical mixtures in line with the policies and procedures established in the statutes administered by the U.S. EPA. They were developed as part of an interoffice guidelines development program under the auspices of the Office of Health and Environmental Assessment (OHEA) in the Agency's Office of Research and Development. They reflect Agency consideration of public and SAB comments on the Proposed Guidelines for the Health Risk Assessment of Chemical Mixtures published January 9, 1985 (50 FR 1170).

These guidelines set forth the principles and procedures to guide U.S. EPA scientists in the conduct of Agency risk assessments, and to inform Agency decision makers and the public about these procedures. In particular, the guidelines emphasize that risk assessments will be conducted on a case-by-case basis, giving full consideration to all relevant scientific information. This case-by-case approach means that Agency

experts review the scientific information on each chemical mixture and use the most scientifically appropriate interpretation to assess risk. The guidelines also stress that this information will be fully presented in Agency risk assessment documents, and that Agency scientists will identify the strengths and weaknesses of each assessment by describing uncertainties, assumptions and limitations, as well as the scientific basis and rationale for each assessment.

Finally, the guidelines are formulated in part to bridge gaps in risk assessment methodology and data. By identifying these gaps and the importance of the missing information to the risk assessment process, the U.S. EPA wishes to encourage research and analysis that will lead to new risk assessment methods and data.

Work on the guidelines began in January 1984. Draft guidelines were developed by an Agency working group composed of expert scientists from throughout the U.S. EPA. The draft was peer-reviewed by expert scientists in the fields of toxicology, pharmacokinetics, and statistics from universities, environmental groups, industry, labor, and other governmental agencies. They were then proposed for public comment. On November 9, 1984, the Administrator directed U.S. EPA offices to use the proposed guidelines in performing risk assessments until final guidelines become available.

After the close of the public comment period, Agency staff prepared summaries of the comments, analyses of the major issues presented by the commentors, and preliminary Agency responses to those comments. These analyses were presented to review panels of the SAB. The guidelines were revised, where appropriate, consistent with the SAB recommendations.

The SAB made several comments and recommendations. Among the recommendations was that the U.S. EPA should develop a separate technical support

document for the mixtures guidelines. The SAB pointed out that the scientific and technical background from which these Guidelines must draw their validity is so broad and varied that it cannot reasonably be synthesized within the framework of a brief set of guidelines. The SAB also identified the need for a technical support document because of the limited knowledge on interactions of chemicals in biological systems and commented that progress in improving risk assessment will be particularly dependent upon progress in the science of interactions. The identification of research needs was an additional SAB concern to be addressed in this support document.

1.2. EXAMPLES OF THE U.S. EPA CHEMICAL MIXTURES RISK ASSESSMENT ACTIVITIES

U.S. EPA personnel were directed by the Administrator to use the guidelines when assessing the human health risks from mixtures of chemicals. They are to be used in developing regulations under the various statutes for pollutants that are mixtures, such as diesel exhaust, coke oven emissions, gasoline and gasoline vapors. Another major use is in assessing the health risks at hazardous waste sites where large numbers of chemicals are frequently encountered.

Many of the statutes that govern U.S. EPA activities suggest a single chemical approach to the regulation of toxic chemicals. For example, the Clean Air Act, Clean Water Act and Safe Drinking Water Act generally instruct the U.S. EPA to protect public health and the environment through regulation of specific sources of pollution or establishment of standards and allowable levels for specific contaminants. In general, when developing regulations to implement these Acts, the U.S. EPA considers the human health hazards of single chemicals.

Some statutes mention chemical mixtures, but generally in combination with the term chemical substance, as in "chemical substance or mixture."

These statutory discussions do not provide one with a clear definition. For example, the Toxic Substances Control Act (TSCA) defines the term "mixture" as follows:

> "The term 'mixture' means any combination of two or more chemical substances if the combination does not occur in nature and is not, in whole or in part, the result of a chemical reaction; except that such term does include any combination which occurs, in whole or in part, as a result of a chemical reaction if none of the chemical substances comprising the combination is a new chemical substance and if the combination could have been manufactured for commercial purposes without a chemical reaction at the time the chemical substances comprising the combination were combined." (TSCA, sec. 3)

Other mixture-related terms are also not clearly defined in the statutes. The term 'hazardous waste' is defined under the Resource Conservation and Recovery Act (RCRA) as a solid waste, or combination of wastes that pose a substantial hazard to human health or the environment when improperly managed. Hazardous wastes encountered at inactive or abandoned facilities or from emergency spill situations are covered under provisions of the Comprehensive Emergency Response, Compensation and Liability Act (CERCLA) and the Superfund Amendments and Reauthorization Act of 1986 (SARA). CERCLA's definition of hazardous substance includes substances and mixtures as defined under a variety of other environmental Acts. For the purposes of this technical support document, definitions for different types of mixtures and mixture interactions are presented later in this chapter.

Perhaps the greatest use of the mixtures guidelines in the U.S. EPA is in assessing human health risk at Superfund sites. These sites generally contain dozens of chemicals in varying concentrations. The Office of Emergency and Remedial Response (OERR) utilizes the risk assessment guidelines, and particularly the mixtures and exposure guidelines in analyzing public health impacts of remedial alternatives at Superfund

hazardous waste sites. OERR's approach is outlined in the Superfund Public Health Evaluation Manual (U.S. EPA, 1986c). The manual covers two elements: baseline evaluations and analysis of remedial alternatives. OERR is currently revising this manual to ensure that it is consistent with the final risk assessment guidelines.

The OERR approach for mixtures is perhaps the most structured of the Agency mixture approaches, involving five specific steps for determining human health risk:

1. Selection of Indicator Chemicals
2. Estimation of Exposure Point Concentrations of Individual Chemicals
3. Estimation of Chemical Intakes
4. Toxicity Assessments
5. Risk Characterization for the Site

An assumption in this process is that there are no data on the specific mixture of concern, or a similar mixture.

The first step is to select a workable number of indicator chemicals. When the number of chemicals found at a site is determined to be too large to work with (>10-15), a scoring system is used to develop a list of indicator chemicals on which to base the assessment. The scoring system considers toxicity information, site concentration data and environmental mobility. Use of professional judgment is encouraged to add or delete chemicals to the list. Indicator scores are used only for relative ranking among the chemicals present and have no meaning outside of the context of the individual chemical selection process. From the indicator scores a smaller, more manageable list of chemicals is selected.

In the second step of this process, baseline environmental concentrations of individual chemicals are estimated using monitoring data and

modeling to estimate when and how human exposures will take place. The Superfund Exposure Assessment Manual describes various chemical fate and transport models that may be used for this step.

The estimation of the amount of human exposure to the selected contaminants is the next step. Concentrations estimated in step two are used to calculate separate intakes for each chemical in each environmental medium: air, groundwater, surface water, fish and soil. These are summed, resulting in total oral exposure and total inhalation exposure. Subchronic and chronic durations are calculated separately. In some cases intake calculations may be based on personal air monitors and body burden data for exposed individuals. Site-specific considerations, such as nonstandard intake values, are considered as appropriate.

In step four, the toxicity information is identified that will be used with results of the exposure assessment in the risk characterization. Toxicity values for chronic and subchronic exposures to noncarcinogens, and carcinogenic potency factors for potential carcinogens are located in available Agency sources. Toxicity data may be developed when necessary. Teratogenic chemicals are listed separately.

The final step involves a comparison between estimated exposures and toxicity values or potency factors. For the noncarcinogenic chemicals, a hazard index is calculated (see Section 5.4) for all chemicals for each medium of exposure. Separate hazard indices, by critical effect, are recommended when the overall hazard index exceeds unity. The mixture guidelines suggest consideration of all types of effects from a particular chemical, not just the "critical effect," i.e., the effect seen at the lowest dose. Critical effect information is readily available in U.S. EPA documentation, while data on other effects may sometimes be more difficult to obtain. For

potential carcinogens, response addition for independently-acting chemicals at low doses is the approach recommended. The manual further assumes that cancer risks are additive across all exposure routes.

Following these five steps, it is recommended that the risk assessor determine the validity of the initial list of indicator chemicals. In addition, a written summary of all the significant uncertainties is recommended as part of the risk characterization step. Assumptions were to have been noted along the way for each step. These public health evaluations are used to develop performance goals and analyses of risks for remedial action alternatives.

Two other approaches for chemical mixtures, relative potency and toxic equivalency factors, have been considered and utilized by U.S. EPA risk assessors and are discussed in Chapter 5 of this document. Briefly, a relative potency method for carcinogenic mixtures is based on the assumption that the ratio of the two potencies is constant, whether it is based on comparisons between human studies, in vivo assays or in vitro assays. The results of human studies are correlated with those of in vivo assays, and results of in vivo bioassays are correlated with the results of in vitro bioassays. The human potency of a poorly-studied mixture can then be estimated from its in vivo (or in vitro) potency multiplied by the potency ratios of a well-studied, similar mixture. The toxic equivalency factor approach has been adopted by U.S. EPA as an interim procedure for estimating risks associated with exposure to chlorinated dioxins and dibenzofurans (U.S. EPA, 1987c). This method relies on in vitro and in vivo data to estimate "toxic equivalency factors" for the various congeners in the mixture. These factors then express the inferred toxicity or cancer risk of poorly studied congeners in terms of the toxicity of a well-studied

congener, and can be used in an additive model to estimate toxicity of a mixture of these congeners.

Many of U.S. EPA's regional offices are routinely using the guidelines, with Superfund activities being the primary application. In addition, at least one region is applying the guidelines in the NPDES permitting program, by using additivity when the pollutants have the same mechanism of action. There are currently programs underway in the U.S. EPA to implement the risk assessment guidelines in all appropriate Agency activities. It will take some time before they are being fully applied in all U.S. EPA operations.

1.3. DEFINITIONS USED IN THIS DOCUMENT

Consistent and clear terminology is critical in the discussion of chemical mixtures risk assessment. Many different definitions have been offered for the terms used with toxicity of chemical mixtures, and most of these are discussed in the body of this document. Except for these historical discussions, the definitions below are used in this document. These definitions are oriented toward their use in risk assessment. For example, the definition of a mixture actually describes "mixed exposures." From a toxicologic standpoint, however, the joint exposures are similar to the single exposure (perhaps time-varying) that would result if the chemicals were physically combined into a true chemical mixture. The following definitions are generally consistent with those found in the literature:

Mixture: Any set of two or more chemical substances, regardless of their sources, that may jointly contribute to toxicity in the target population.

Simple Mixture: A mixture containing two or more identifiable components, but few enough that the mixture toxicity can be adequately characterized by a combination of the component toxicities.

Complex Mixture: A mixture containing so many components that any estimation of its toxicity based on its component toxicities contains too much uncertainty and error to be useful. The chemical composition may vary unpredictably over time or with different conditions under which the mixture is produced. Complex mixture components may be generated simultaneously as by-products from a single source or process, intentionally produced as a commercial product, or may co-exist because of disposal practices. Risk assessments of complex mixtures are preferably based on toxicity and exposure data on the complete mixture. Gasoline is an example.

Similar Mixtures: Mixtures having the same components but in slightly different ratios, or having most components in nearly the same ratios with only a few different (more or fewer) components, and displaying similar types and degrees of toxicity. Diesel exhausts from different engines are an example of similar mixtures (Appendix B).

Chemical Classes: Groups of compounds that are similar in chemical structure and biological activity, and which frequently occur together in the environment, usually because they are generated by the same commercial process. The composition of these mixtures is often well controlled, so that the mixture can be treated as a single chemical. Polychlorinated biphenyls (PCBs) are an example.

Interaction: The circumstance in which exposure to two or more chemicals results in a qualitatively or quantitatively altered biological response relative to that predicted from the actions of the components administered separately. The multiple chemical exposures may be simultaneous or sequential in time and the altered response may be greater or smaller in magnitude (adapted from NRC, 1980). For quantitative evaluations, the "no-interaction" prediction is based on dose or response addition, as appropriate.

Synergism: A response to a mixture of toxic chemicals that is greater than that suggested by the component toxicities.

Antagonism: A response to a mixture of toxic chemicals that is less than that suggested by the component toxicities.

Potentiation: A special case of synergism in which one substance does not have a toxic effect on a certain organ or system, but when added to another chemical it makes the latter much more toxic.

Inhibition: A special case of antagonism in which one substance does not have a toxic effect on a certain organ or system, but when added to a toxic chemical it makes the latter less toxic.

Masking: The situation in which the toxic effect of one chemical is not displayed because of functionally competing effects from the other chemical. The most striking example is when the carcinogenic activity of the mixture is not observed at the experimental doses, because of more obvious toxic signs, particularly mortality, induced by other toxic components.

1.4. OVERVIEW OF THIS DOCUMENT

The main body of this report discusses the information available on chemical mixtures, the mechanisms by which chemicals interact, and the mathematical models used to describe toxicant interactions. After a brief initial description of the terminology used to describe toxicant interactions, Chapter 2 discusses the nature of the available information on three general categories of mixtures: complex mixtures, mixtures composed of a single class of chemicals and simple mixtures. This section is intended to illustrate the differences between the types of information that are available on the various categories of mixtures but is not intended to be a compendium of all available information on all mixtures. Emphasis is placed on the description of the tests used to assess the toxicity of the mixture as well as the available methods and feasibility of these methods for quantitatively measuring interactions of the components in the mixture. This chapter concludes with discussions of additional topics: interactions of carcinogens with other compounds, some results from the Agency's data base on mixtures and quantitative measures of interactions.

A discussion of mechanisms of toxicant interactions is presented in Chapter 3. This section discusses the ways in which compounds may interact: direct chemical-chemical reactions that result in the formation of a different chemical species as well as the biological bases of toxicant interactions such as effects on absorption, distribution, metabolism, excretion and receptor site affinity. This is followed in Chapter 4 by a review of the mathematical models and statistical procedures used to assess toxic

interactions, including dose addition, response addition, generalized linear models, and response surface models. This section concludes with a critical review of statistical methods used in research articles that are covered in the Agency's mixtures data base.

Chapter 5 reassesses the guidelines in terms of the information summarized in the previous chapters. Following the organization of Chapter 2, which is in turn dictated by the different types of information available on the various chemical classes, this chapter separately discusses complex mixtures, similar mixtures and simple mixtures. For complex mixtures, emphasis remains on *in vivo* bioassays, the applicability of which can be extended by the concept of sufficient similarity, as illustrated in Appendix B. Recognizing the highly variable nature of some complex mixtures as well as the difficulty and expense of obtaining good *in vivo* bioassays, the relative potency method, the "toxic equivalency factor" method and analogous methods based on *in vitro* assays, are more strongly endorsed than in the original guidelines. A limitation of dose addition is also discussed, primarily related to limitations of risk assessment of single compounds.

This document concludes with a brief outline of research needed to improve or validate the risk assessment procedures for mixtures. Because the reassessment of the guidelines relies heavily on the use of *in vitro* tests, emphasis is placed on the validation of such tests using whole animal assays.

2. TYPES OF INFORMATION AVAILABLE

2.1. OVERVIEW

This chapter summarizes the kinds of information available on various categories of mixtures; namely, complex mixtures, chemical classes and simple mixtures. Also covered is the nature and utility of information available on the interactions of carcinogens with other compounds including discussions of promotion, cocarcinogenicity, inhibition and masking. The focus of this chapter is on the usefulness as well as the limitations of available data on mixtures for risk assessment. This is not intended to provide a comprehensive summary of all available information on these topics but is based on the information included in the computerized data base, which is described in Section 2.4.3., Chapter 4 and Appendix A.

Given the quality and quantity of the available data on chemical interactions, few generalizations can be made concerning the likelihood, nature or magnitude of interactions. Most interactions that have been quantified are within a factor of 10 of the expected activity based on the assumption of dose addition. The limited available information suggests that at least some interactions may have thresholds and that additivity may be a plausible assumption at low levels of environmental exposure. This supposition is reenforced by mechanistic considerations discussed in Chapter 3. It must be emphasized, however, that these generalizations are based on very limited data.

The information available on complex mixtures is fundamentally different in design and focus from that on simple mixtures. Studies on complex mixtures generally are designed to characterize the toxic properties or potency of the mixture as an entity. In this respect, the design and conduct of such studies do not differ greatly from studies on single

compounds. As a consequence, the great majority of the bioassays on complex mixtures are not useful for assessing potential interactions of components in the mixtures. In some cases, however, sample collection or concentration of complex mixtures prior to a bioassay may cause changes in the composition of the mixture, which could limit the applicability of the study in risk assessment. This factor, however, is not greatly different from problems that can be encountered in the preparation and purification of a single compound prior to bioassay.

Studies available on simple mixtures are generally restricted to binary combinations and are usually designed to measure the magnitude of the interaction among the components in the mixture. The study design generally includes a control group, one or more groups of subjects exposed to each component of the mixture at one or more dose levels, and one or more groups exposed to one or more doses of all components at equal ratios. The interaction is generally reported as the ratio of the observed response to a response predicted by the assumption of dose additivity (discussed in Chapter 4).

Studies on chemical classes are generally similar to those on complex mixtures. For instance, most of the available information on mixtures of polychlorinated biphenyls (PCBs) comes from bioassays on commercial mixtures of these substances, and no quantitative measures have been attempted of the individual components as to their concentration or biological activity. A significant amount of information is available on individual components of many complex mixtures and chemical classes, but such studies are not directly useful in quantifying interaction.

The restriction to binary mixtures of bioassays that attempt to quantify mixture interaction, and the virtual absence of bioassays on complex

mixtures or mixture classes that attempt to define such interactions, is attributable to the nature of the experimental design that is necessary for quantifying interactions. Note the example given by Clayson (1984):

> "... if it was wished to examine the interactions of just 10 chemicals in pairs it would involve conducting 45 separate bioassays plus a further 10 for the single chemicals. If it was deemed necessary to study these pairs of chemicals in just 5 different ratios it would be necessary to undertake 255 separate bioassays. As there are estimated to be in excess of 25,000 chemicals produced commercially in significant quantities, examination even in pairs becomes quite impracticable with about 313 million tests if only one ratio is used or 1.57 American billion tests [sic] if 5 different ratios were employed."

The difficulties in obtaining quantitative measures on toxicant interactions are exacerbated by the fact that many of the studies on binary mixtures that purport to quantify toxicant interactions are improperly designed and the reported results are either uninterpretable or are difficult to compare among different studies.

Studies on the interactions of carcinogens with other compounds share many of the same difficulties and limitations as those discussed above. A substantial body of data, however, has accumulated which suggests that some compounds may markedly modify the carcinogenic potency of other compounds. Although the early investigations focused on dermal applications and enhancement of skin tumor response, more recent studies indicate that such interactions may be relatively common and affect cancer induction at other sites. Conversely, some agents are known to inhibit the carcinogenicity of other compounds. The inhibitory activity of some materials can vary as a function of time of application in relation to the carcinogen as well as the tumor site.

2.2. COMPLEX MIXTURES

2.2.1. Overview. Some classes of chemical mixtures, such as automotive emissions and coke oven emissions, are composed of hundreds of components produced by a single process or set of related processes. Some of the components may be grouped into similar classes while others may not have any apparent structural or toxicologic similarity to other elements of the mixture. While toxicologic data may be available on some of the mixture components or classes of components, the characterization of the toxicity of other agents in the mixture may be incomplete or nonexistent. In addition, the chemical composition of such mixtures may vary over time or as a function of changes in conditions (e.g., temperature or pressure) under which the mixtures are generated. For example, it has been demonstrated that malfunctioning fuel injection systems in diesel engine cars can cause increased mutagenicity and benzo[a]pyrene emissions (Zweidinger, 1982). As is the case for data on individual toxic agents, the quality and quantity of data on complex mixtures varies markedly among different mixtures. Few generalizations can be made concerning the nature of the available data or the applicability of these data for use in risk assessment.

2.2.2. Epidemiologic Studies. In a few instances, human exposures to complex mixtures have been sufficiently high that direct human data are available for quantifying risks from exposure to the mixtures or processes generating the mixtures. This has most often been the case for mixtures that induced cancer. For instance, a substantial body of epidemiologic literature is available on the carcinogenic potency of cigarette smoke and of coke oven emissions. Such epidemiologic investigations, while sometimes allowing for quantifying of risk from exposure to the complex mixture, seldom provide information on the nature, magnitude or significance of

interactions among the components in the mixture. Some interactions involving exposure to complex mixtures that have been quantified include those between cigarette smoke and asbestos (Hammond and Selikoff, 1973; Hammond et al., 1979; and Selikoff et al., 1968), cigarette smoke and radiation exposure (Lundin et al., 1969), as well as cigarette smoke and vitamin A (Dayal, 1980). Even these examples, however, which are the best studied examples providing human data on interactions involving complex mixtures, do not quantify interactions among components in the complex mixture but rather measure interactions between the complex mixture and another agent.

In 1981, a WHO committee on health effects of combined exposures in the work environment concluded the following: "The dearth of sound epidemiological studies to date and the potential importance of at least some of the possible interactions between occupational and nonoccupational environmental factors attest to the need for more work in this field" (WHO, 1981). The more recent literature (e.g., Kopfler and Craun, 1986; WHO, 1983) has not substantially improved the prospect of developing human data on complex mixtures that will be useful in quantifying component interactions. Given the difficulties in assessing and designing studies to measure interactions in simple binary mixtures [as discussed in general in Section 2.4.1. and discussed specifically in terms of epidemiologic studies by Andelman and Barnett (1986)], human data on complex mixtures are likely to remain most useful for risk assessments on the complex mixture itself but will seldom if ever be adequate for the quantitative assessment of interactions among components within the mixture.

2.2.3. Whole Animal Bioassays. For most groups of highly complex mixtures, data on whole animal bioassays that are directly useful for risk assessment are not available. Lewtas (1985), for example, has reviewed the

available data on combustion emissions from diesel engines, gasoline engines, and energy combustion sources (wood stoves, oil furnaces, and utility power plants). For the gasoline and diesel engines, the most comprehensive in vivo data are from mouse skin tumor initiation studies, which are usually not directly used in risk assessment to estimate carcinogenic potency in humans. While several in vivo studies have examined the carcinogenic and systemic effects of diesel exhaust, the data base, including epidemiologic data, in general, is extremely limited (NAS, 1981). For the energy combustion sources, no in vivo studies are available. A large body of data, however, is available on these and other mixtures using a variety of in vitro test systems. This information is discussed in Section 2.2.4. below, and the potential use of these data in quantitative risk assessment of mixtures is discussed in Chapter 5.

Although data from animal studies are available for the few complex mixtures that have been identified as human carcinogens, long-term in vivo animal bioassays on complex mixtures have tended to follow rather than lead epidemiologic investigations and have focussed on complex mixtures such as polycyclic aromatic hydrocarbons (PAH), coke oven emission, and diesel exhaust (as discussed in Appendix B) for which data on human effects or human exposures suggested a potential hazard. The paucity of whole animal bioassay data on complex mixtures is illustrated by the compilation of cancer risk assessments currently on the U.S. EPA's Integrated Risk Information System (IRIS). Of the 95 risk assessments currently on IRIS one is for a technical grade mixture of hexachlorocyclohexane isomers, one is for a binary mixture of hexachloro-p-dioxins and one is for mixtures of xylene isomers. Only two assessments, nickel refinery dust and creosote, are for complex mixtures. The assessment of one of these mixtures, nickel

refinery dust, is based on epidemiologic data rather than animal bioassay data (U.S. EPA, 1987b). Similarly, although the International Agency for Research on Cancer has identified several industrial processes that involve exposure to complex mixtures and which are causally associated with cancer in humans based on epidemiologic studies, no complex mixtures have been designated as carcinogens based solely on the results of animal bioassays (IARC, 1982).

As is the case for epidemiologic investigations, long-term whole animal bioassays on complex mixtures can be useful for risk assessments on the complex mixture itself but are not, and from a practical perspective cannot, be designed for the quantitative measurement of interactions among components within the mixtures. The practical difficulties in making such measurements for complex mixtures are an extension of those discussed for binary mixtures in Section 2.4.1. In addition, because of the variability of complex mixtures over time or with different conditions in the generation of the mixture, the few bioassays that are available on complex mixtures are not necessarily applicable to all exposures to the complex mixture. This is illustrated in Appendix B for diesel exhaust.

Several short-term in vivo assays for carcinogenic activity such as the mouse skin initiation/promotion assay (Pereira, 1982a; Slaga et al., 1982), rat liver focus bioassay (Herren-Freund and Pereira, 1986; Pereira, 1982b), and strain A mouse lung tumor bioassay (Maronpot et al., 1986; Stoner and Shimkin, 1982) have been developed for assessing the effects of mixtures. Such studies are normally not used as the sole basis for a quantitative risk assessment because of the relatively short periods of exposure and the endpoints that are measured. Nonetheless, because these studies can be conducted more rapidly and less expensively than standard chronic bioassays,

they can be applied in qualitative or quantitative assessments of interactions. Such short-term in vivo tests more closely approximate the chronic in vivo assays that are normally used in risk assessments and thus may have more intuitive appeal than in vitro assays. Nonetheless, comparative analyses between the results of such short-term in vivo assays with other short-term assays (Pereira and Stoner, 1985) or long-term in vivo bioassays (Herren-Freund and Pereira, 1986) do not clearly indicate the such assays are superior to some of the in vitro assays discussed below. The short-term in vivo assays that have been developed to date focus only on sceening tests for carcinogenic activity. Research articles describing comparable tests for measuring interactions in the induction of chronic toxic effects have not been located.

2.2.4. In vitro Studies and Other Screening Tests. Certain aspects of the toxicity of complex environmental mixtures have been evaluated extensively using in vitro assays and other screening tests. Four types of assays have been most often used: microbial mutagenicity, cell culture, embryo bioassays and plant cytogenetics. The endpoints assessed in these assays are one or more of genotoxicity, cytotoxicity, embryotoxicity and impaired development. Although the utility of many of these assays in quantitatively or qualitatively assessing the in vivo biological activity of single compounds or complex mixtures has not been extensively validated (as discussed in Section 5.1.), these in vitro assays are currently the only practical approach to obtaining detailed information on the biological activity of complex mixtures, particularly in site-specific and process-specific assessments.

The Salmonella histidine auxotroph reversion assay (Ames et al., 1975) has been the most widely used procedure for detection of mutagenicity of complex mixtures. Numerous environmental mixtures, as entities or after

fractionation, have been tested in this assay: coal-liquification and gasification products (Epler et al., 1978; Rao et al., 1980; Schoeny et al., 1981; Houk and Claxton, 1986), automotive and diesel exhaust (Huisingh et al., 1978; Claxton and Kohan, 1980), crude shale oil (Epler et al., 1978), drinking water (Chriswell et al., 1978; Loper and Lang, 1978), cigarette smoke (Kouri et al., 1978), industrial effluents (Commoner et al., 1978; Douglas et al., 1983; McGeorge et al., 1984), urban ambient air particulate and extracts (Commoner et al., 1978; Butler et al., 1984), sludge (Houk and Claxton, 1986) and waste-amended soil (Donnelly et al., 1983). Mutagenic activity of mixtures has also been assessed in a forward mutation in Salmonella typhimurium using 8-azaguanine resistance for selection. Automotive exhaust (Claxton and Kohan, 1980), oil shale and water samples (Whong et al., 1983) and coal liquefaction products (Schoeny et al., 1986) have produced positive results in this assay.

Fractionation, or separation of the mixture into chemically-related or distinct constituents, has been utilized to define constituents in a mixture more clearly and to determine which compounds are responsible for mutagenic activity. Fractionation procedures have also been used to concentrate materials and to reduce toxicity of whole mixtures, thus making them more amenable to assay. Extraction methods (e.g., acid/base, polar/nonpolar), however, may lead to chemical reactions that could alter the components of the mixture, thereby affecting the toxicity.

The application of reconstruction assays can be useful in assessing the effects of fractionation procedures or in uncovering interactions among the fractions. Thilly et al. (1983), for example, identified the relative abundance of constituents in particulates from kerosene combustion (kerosene soot). The mutagenic contribution of the 14 most abundant compounds was

determined in the Salmonella forward mutation assay. When these chemicals were combined in appropriate proportions to approximate the "pure soot," the mutagenicity of the reconstituted kerosene soot was equivalent to the original soot extract, demonstrating the concentration dependence of mutagenicity for the mixture, that is, additivity. By contrast, a similar study of fractionated coal hydrogenation materials in which the sum of the mutagenic activities of organic extracts was compared with the activity from the whole sample and with a reconstituted whole sample, indicated a departure from additivity for some mixtures (Schoeny et al., 1986).

DNA repair-deficient strains of Bacillus subtilis (Donnelly et al., 1983) and Escherichia coli (Rossman et al., 1984) have been used to detect alterations in DNA induced by wood-preserving waste and by urban air particulates, respectively. Assays for reverse mutation in Saccharomyces cerevisiae (yeast) (Douglas et al., 1983) and assays detecting dominant or recessive lethals in Paramecium tetraurelia (Smith-Sonneborn et al., 1983) have been less frequently used.

Embryo culture assays have been developed to examine potential embryolethality, malformation and growth/developmental alterations induced by individual substances and complex environmental mixtures. Dumont et al. (1983) developed the Frog Embryo Teratogenicity Assay: Xenopus or FETAX. Coal and shale-derived synfuels (Dumont et al., 1983) and mine water discharge (Dawson et al., 1985) have caused one or more of embryolethality, gross malformation or embryotoxicity in frog embryos exposed in vitro. Other embryo assays using the rat (Klein et al., 1983) and sea urchin (Hose, 1985) are being developed and validated for application to mixtures.

Environmental mixtures have been evaluated in cell culture assays as to their potential mutagenicity at specific loci, as well as for their capacity

to induce sister chromatid exchange or chromosomal aberrations. Cytotoxicity has also been evaluated as measured by effects on cellular growth and division, and on morphological, cytochemical and biochemical alterations. For example, Chinese hamster ovary (CHO) cells have been used in determinations of the ability of complex environmental mixtures to produce cytotoxicity and mutagenicity at the hypoxanthine guanine phosphoribosyl transferase locus (Hsie et al., 1978). Subfractions of crude synthetic oil (Hsie et al., 1978), coal gasification condensate tar (Cunningham et al., 1984), oil and coal fly ash (Chescheir et al., 1980; Li et al., 1983), textile mill effluents (Waters et al., 1978), diesel engine exhaust (Chescheir et al., 1980; Li et al., 1983), retort process water from crude shale oil (Strniste et al., 1983), as well as coke oven emissions, roofing tar and cigarette smoke condensate (Li et al., 1983), have produced positive cytotoxic and mutagenic responses in this assay.

Other endpoints measured in CHO cells in response to complex environmental mixtures have included mutagenicity at the Na^+-K^+-dependent ATPase locus using ouabain resistance for selection, sister chromatid exchange and chromosomal aberrations. Diesel exhaust particle extract (Li et al., 1983) and pulp and paper mill effluents (Douglas et al., 1983) were genotoxic in these assays.

Cell types such as alveolar macrophages or epithelial tissue, which would be directly exposed to environmental agents, have been used to evaluate toxicity of mixtures. In the pulmonary alveolar macrophage assays, viability, phagocytic ability, specific enzyme activities and ATP levels are the endpoints most often evaluated. The toxicity of <u>in vitro</u> exposure to various fly ash particles (Waters et al., 1978; Aranyi et al., 1980; Mumford and Lewtas, 1983), liquid textile mill effluents (Waters et al., 1978) and

smelter dust (Aranyi et al., 1980) was assessed using rabbit alveolar macrophages. Fisher et al. (1983) used bovine alveolar macrophages for the analysis of coal and oil fly ash. Unscheduled DNA synthesis, an indication of DNA damage, was induced in organ cultures of hamster tracheal epithelium exposed to coal-fired fly ash, diesel fuel exhaust and cigarette smoke condensate (Schiff et al., 1983).

Other less frequently used cell culture assays have been applied to environmental mixtures. The BALB/c-3T3 cell transformation assay showed enhanced toxicity of drinking water organic concentrate fractions (Loper and Lang, 1978). The rainbow trout gonadal tissue (RTG-2) assay, wherein anaphase aberrations resulting from in vitro exposure are determined, showed genotoxicity of marine sediment samples (Kocan and Powell, 1984).

In vitro plant assays have been used to evaluate various environmental mixtures. Plants, like animals, are eukaryotic organisms and may have the ability to convert chemical compounds to biologically active species. The most widely used higher plant for testing genetic toxicity has been Tradescantia. Tradescantia plant systems are especially useful for in situ environmental air exposure and the testing of gaseous agents. The induction of somatic mutation at a particular locus is measured in the Tradescantia stamen hair system as a phenotypic change in pigmentation in mature flowers following exposure of the developing floral tissue (Schairer et al., 1978, 1983). Tradescantia exposed in situ for 10 days to ambient air pollution in several cities in the United States have shown positive results for mutagenicity in this assay (Schairer et al., 1978, 1983). In the Tradescantia micronucleus test, early prophase I meiotic pollen mother cells of Tradescantia plant cuttings are exposed and the frequency of micronuclei (chromosomal fragments) determined in the tetrads following meiosis (Ma et al., 1980, 1983; Plewa, 1984). Sewage sludge from several cities (Plewa, 1984),

shallow well water samples and deep well water containing ^{226}Ra, as well as combustion products of diesel and diesel/soybean oil fueled engine exhaust fumes (Ma et al., 1980, 1983, 1984) were genotoxic in this assay.

The barley root tip cytogenetic system involves scoring barley (Hordeum vulgare) root tip cells in germinating seeds at anaphase for detectable aberrations following treatment of the seed (Constantin et al., 1980). Fly ash-aqueous extracts and arsenic-contaminated groundwater have produced positive results in this assay. The Arabidopsis thaliana assay (Redei, 1980) and the Soybean Spot Test using Glycine max (Vig, 1980), while not yet applied to complex environmental mixtures, detect phenotypic alterations in the embryo or mature plant indicative of mutational events occurring as a result of exposure of the seed.

2.3. MIXTURES OF CHEMICAL CLASSES

A mixture of a class of chemicals refers to a group of compounds that are structurally and biologically similar and which usually occur together in the environment because they are produced by the same process. Mixtures of chemical classes, like the complex mixtures, may contain tens or hundreds of components. Also, as with the complex mixtures, the composition of similar mixtures may vary over time because of environmental partitioning or different conditions of generation, use and release. Examples of mixtures of chemical classes include the chlorinated dioxins, chlorinated dibenzofurans, chlorinated naphthalenes and chlorinated biphenyls.

As with the complex mixtures, the amount of data available on mixtures of chemical classes varies markedly, but the types of data are similar: human data (generally data from accidental exposures), animal bioassay data, and data from in vitro assays. The relative amounts of the various types of data are dependent on the levels and nature of human exposure to the

mixtures, the perceived levels of hazards associated with exposure to each mixture, and certain practical considerations that are associated with some of the more common simple mixtures.

For instance, PCBs have been commercially produced as several groups of similar mixtures varying in the average degree (percent by weight) of chlorination (U.S. EPA, 1984). For the more commercially significant PCB mixtures, such as Aroclor 1254 (54% Cl) and 1262 (62% Cl), whole animal bioassays for carcinogenic effects are available on the mixture and have been used directly to estimate cancer potency. The chlorinated dioxins, however, have never been used as a commercial product but have occurred as contaminants in commercial products or as combustion by-products (U.S. EPA, 1985). Consequently, there is no "typical" dioxin mixture, and whole animal bioassays have been conducted only on certain individual dioxins (such as 2,3,7,8-tetrachlorodibenzo-p-dioxin) or on simple mixtures of hexachlorinated dioxins, which are difficult to separate chemically. Given technical problems associated with the synthesis and purification of large quantities of chlorinated dioxins as well as the undesirability of synthesizing large quantities of them, it is not likely that many more whole animal bioassays will or should be conducted on this class of chemicals. Much research, however, has been and continues to be conducted using *in vitro* bioassays to facilitate a better understanding of structure-activity relationships and mechanisms of action of chlorinated dioxins as well as many other classes of simple mixtures. These data have been recently reviewed (Kociba and Cabey, 1985) and their application to risk assessment is an active topic in the literature (U.S. EPA, 1987c; Eadon et al., 1986) and is discussed further in Chapter 5.

2.4. SIMPLE MIXTURES, COMPONENTS AND TOXIC INTERACTIONS

2.4.1. Overview. The great majority of studies in which attempts have been made to assess toxic interactions quantitatively have used simple binary mixtures. Only a few studies (Gullino et al., 1956; Hermens et al., 1985a,b) have used mixtures of over 10 compounds. In such studies of relatively simple mixtures, approaches to the analysis of toxicant interactions used by most toxicologists have been based on the assumption of dose addition using simple experimental designs involving a control group, groups exposed separately to each compound at multiple doses so that the LD_{50}s or ED_{50}s can be estimated, and groups exposed at multiple doses to one mixture of all compounds in fixed proportions. The degree and nature of the toxic interaction is then expressed as the ratio (or some transformation of the ratio) of the observed LD_{50} or ED_{50} of the mixture to the LD_{50} or ED_{50} expected from the assumption of dose addition. This can be referred to as the ratio of interaction (R.I.) and expressed as

$$R.I. = ED_{50}(Obs)/ED_{50}(Exp) \qquad \text{Equation 2-1}$$

A ratio of interaction greater than one is associated with antagonism in that the observed ED_{50} is greater than expected (i.e., less toxic) based on the assumption of dose additivity and the measured ED_{50}s of the components in the mixture. Conversely, a ratio of less than one is associated with synergism.

As discussed by Berenbaum (1981, 1985a) and detailed in Chapter 4, the difficulty in demonstrating significant interaction based on studies using single ratios of interaction is primarily one of experimental design. Since the ratio of interaction is dependent on the proportions of the components in the mixture, a test has the best chance of demonstrating significant interaction if the mixture giving maximum interaction is selected. If the

combination of toxicants being tested is assumed to evidence a pattern of symmetric interaction, a mixture of equitoxic doses would be the best selection. Even with this simplifying and not necessarily valid assumption, however, tests based on single ratios of interaction will not yield significant results unless the magnitude of the interaction is substantial and the experimental variability is minimal.

2.4.2. Measurements of Toxicant Interactions. Keplinger and Deichmann (1967) used the ratio of interaction to measure the joint action of various pesticides in mice. In this study, only one mixture of each combination was used, and significant interaction was arbitrarily defined as ratios of <0.57 for synergism and >1.75 for antagonism. Smyth et al. (1969, 1970) used a slightly modified expression of the ratio of interaction, which resulted in estimates that resembled the shape of a normal distribution. Significant interaction was then defined as those ratios of observed to predicted LD_{50}s in rats that were beyond 1.96 standard deviations from the mean ratio. In studies on the joint action of pesticides in houseflies, Sun and Johnson (1960) defined the cotoxicity coefficient as the ratio of interaction multiplied by 100. Significant interaction was estimated in this study by taking repeated measurements and determining if the 95% confidence interval of the cotoxicity coefficients included 100. They reported a high degree of synergism for a mixture of 8-(dimethoxyphosphinyloxy) N,N-dimethylcrotonamide and sesamex while methylparathion and sesamex were antagonistic. Wolfenbarger (1973) used cotoxicity coefficients to estimate the joint action of toxaphene-DDT mixtures in insects. Although different combinations of each mixture were used and cotoxicity coefficients were derived for each combination, no attempt was made to derive coefficents of interaction, as defined and discussed in Section 4.3., which could be used

to characterize the direction and magnitude of the interaction for all combinations of the mixtures.

All of the above approaches are severely limited by their reliance on a single interactive ratio. As discussed by Hewlett (1969), the ratio of interaction is characteristic only of a particular combination of compounds. In other words, the estimated value of the ratio of interaction will vary depending on the proportions of the toxicants present in the mixture.

Another limitation in the use of ratios of interaction is encountered in attempts to demonstrate statistical significance. The method used by Sun and Johnson (1960), based on repeated measurements of the ratio of interaction, may be the least objectionable; however, because of the dependence of the ratio of interaction on the proportion of the components in the mixture, the estimate of interaction is valid only for the particular mixture tested and has no merit in assessing the overall interaction characteristic of the combination being tested. This limitation may be particularly misleading for those compounds that evidence asymmetric interaction, as discussed in Chapter 4. The approach adopted by Keplinger and Deichmann (1967) is totally arbitrary and makes no attempt to establish a criterion for statistical significance. The method of Smyth et al. (1969, 1970) is based on arbitrary selection of test chemicals that influence the criteria for interaction. The other methods that use 95% confidence intervals of the LD_{50}s of the mixture and individual components (Marking and Dawson, 1975; Wolfenbarger, 1973) are overly sensitive to both endogenous and exogenous variance. Marking and Dawson (1975) recognized the difficulty with exogenous variance in stating that "well planned toxicity tests which result in narrow confidence intervals are most useful in the assignment of the effects of chemical mixtures." If endogenous variation is high (that is,

the slope of the log dose-probit response line is low), however, even well-designed toxicity tests may yield 95% confidence intervals that preclude the detection of interaction.

2.4.3. The U.S. EPA Data Base on Toxic Interactions. The interaction data base was constructed to determine the nature and extent of information on component interactions. Most of the entries are for studies on two chemical interactions, but a few consider combinations of two mixtures. The data base currently covers roughly 600 chemicals. Most of the studies evaluate the interactions based on mortality following acute exposure. Most of the studies investigate the influence of one chemical on the toxicity of another (i.e., potentiation or inhibition), where the first is administered at a nontoxic dose (Table 2-1). The statistical methods used in these studies are discussed in Chapter 4. Details of the data base are given in Appendix A.

2.5. INTERACTIONS OF CARCINOGENS WITH OTHER COMPOUNDS

2.5.1. Promoters and Cocarcinogens. Only 13 years after Bauer (1928) proposed the somatic cell mutation theory of cancer, Rous and Kidd (1941) and Berenblum (1941a) proposed that some forms of chemically induced cancers involved a two step process. Berenblum's (1941b) report on the enhancement of benzo(a)pyrene induced carcinogenicity by extracts of Croton tiglium, a complex mixture, was the first example of one chemical enhancing the carcinogenic activity of another. With improvements in chemical techniques for fractionation and isolation, the active components of Croton resin have been identified (Hecker, 1968; Van Duuren, 1969). Since 1941, over 30 such agents, including all extracts or derivatives from Croton oil, have been identified (Van Duuren, 1976; Pitot and Sirica, 1980; Fujiki et al., 1979). The best known of these is TPA (12-o-tetradecanoyl-phorbol-13-acetate).

TABLE 2-1

Summary of Interaction Data Base

Category	Type	Percentage of Studies*
Duration	acute	73
	subchronic	11
	chronic	8.4
	lifetime	0.29
Interaction	synergism	2.8
	potentiation	29
	antagonism	1.7
	inhibition	31
	additivity	3.7
	no apparent interaction	25
	masking	0.59
	chemical synergism	0.13
	unable to assess	5.6

*Representing a total of 587 chemicals

The extensive and complex literature on promotion and cocarcinogenicity has been recently reviewed by Bohrman (1983), Clayson (1984), Driver and McLean (1986). For purposes of this document, promoters will be defined as agents which, when applied after but not before an initiator, act to enhance the carcinogenicity of the initiating agent. Cocarcinogens are taken to be agents that may enhance carcinogenicity when applied before or at the same time as the initiator. The definitions of cocarcinogens and promoters are, thus, operational and depend largely on the design of the experiments in which they are found to have an effect. It is likely that cocarcinogens and promoters may have some mechanisms of action in common, as well as some unique modes of enhancing a carcinogenic response.

As discussed by Van Duuren (1976), all promoters can probably display at least some tumorigenic activity in the absence of a known initiator. This is to be expected "... if one assumes that in any group of animals there will be some that have latent tumor cells, either by earlier exposure to an external initiating agent or by spontaneous conversion of normal cells into latent tumor cells... If this explanation is accepted, the question about 'pure' promoting agents should be obsolete." While this may be true within the context of interpreting the results of an initiation-promotion assay, the distinction between promoters and initiators could have a significant impact on risk assessment. Because it is generally accepted that initiation is a nonthreshold (genotoxic) phenomenon and promotion is probably a threshold (epigenetic) phenomenon, the distinction between "pure" promoters and those promoters that may also be weak initiators may be crucial to the selection of high- to low-dose extrapolation models, as discussed further by Clayton (1984) and in Section 3.3.6. and 5.5.

While most of the studies on chemical promotion involve dermal or oral exposure, Nettesheim et al. (1981) documented several factors enhancing

carcinogenesis in the respiratory tract; at least some of these were attributable to initiation-promotion processes (e.g., promotion of urethane-induced pulmonary tumors in mice by phorbol esters or butylated hydroxytoluene). In addition to the skin and respiratory tract, initiation-promotion has also been observed in the liver (2-AAF- or DMN-phenobarbital), bladder (N-methyl-N-nitrosourea-sodium saccharin or cyclamate), gastrointestinal tract (DMBA-TPA, dimethylhydrazine-phenobarbital), and mammary glands (DMBA-estrogens or phorbol esters), as detailed in an extensive review by Bohrman (1983). Some epidemiologic data are suggestive of a two-stage initiation-promotion process in humans, although the evidence is scanty (Hakama, 1971; Berenblum, 1979). Thus, promotion may be a very common phenomenon that occurs among many chemicals and affects most species.

2.5.2. Inhibitors and Masking. Some compounds, such as butylated hydroxytoluene (BHT) and other antioxidants, have been shown to decrease the development of tumors when administered before the administration of known carcinogens (Ito et al., 1985; King and McCay, 1983; NRC, 1980). In addition, a compound that causes an increase in the mortality rate could result in a decreased cumulative incidence of late appearing tumors because of competing risks.

In the case of compounds that apparently decrease carcinogenic response through a "protective" mechanism, the nature of the protective mechanisms and the dose-response relationship of the protective effect have not been clearly defined. In addition, some of the compounds that display a "protective" effect under one set of circumstances may, in fact, enhance the carcinogenic response under different conditions of exposure. For instance, BHT reduces carcinogenic responses when administered before some carcinogens but enhances carcinogenic responses when administered after other

carcinogens. The protective effect is attributed to the antioxidant properties of BHT and the enhancement to production of a metabolite of BHT with promoting activity. Any attempt to predict the interaction of BHT with a specific carcinogen is complicated because BHT is known to inhibit the mutagenic activity of benzo[a]pyrene but to enhance the mutagenic activity of aflatoxin B1 in the Salmonella reverse mutation assay (Malkinson, 1983). The sequence of exposure is an important variable for other compounds as well. Both phenobarbital and clofibrate, for example, enhance carcinogenic response when administered subsequent to an initiator. When administered concurrently with an initiator, however, phenobarbital inhibits tumor formation whereas clofibrate enhances tumor formation (Mochizuki et al., 1983). In addition to variations in the effects of dose schedule on carcinogenic interaction, the nature of the interaction may also vary with the site of action. For example, Anderson et al. (1983) have noted that PCBs (Aroclor 1254) inhibit the development of lung tumors but enhance the development of liver tumors in mice initiated with N-nitrosodimethylamine.

As with the quantifying of cocarcinogenicity and promotion, a consistent and predicable pattern of interaction has not yet emerged in the assessment of compounds that inhibit carcinogenicity (Schulte-Herman, 1985; Williams, 1984). Until such a pattern does emerge, it is not likely that studies such as those described above will be used to modify quantitative risk assessments for chemical mixtures.

Conversely, both inhibition and masking may be significant in the interpretation of cancer bioassay data on mixtures. For instance, Raabe (1987) has recently presented an analysis of the dose-time-response relationships of plutonium-239 in causing deaths from pneumonitis and lung cancer in beagle dogs. Deaths from pneumonitis tended to occur at higher doses and earlier in life than deaths from cancer, thus masking the carcinogenic

activity seen at lower doses. Similar patterns have been seen in the results of many cancer bioassays on single compounds in which early mortality from causes other than cancer confounded the interpretation of the results. For bioassays on mixtures of compounds, the results of masking of carcinogenicity because of early mortality could be particularly significant if the mixture contains known carcinogens. For example, human exposure to the mixture at concentrations below the toxic threshold could result in a significant increase in the risk of cancer that would not be reflected in the animal bioassay. No data, however, were located that specifically address this issue in the published literature.

2.6. QUANTIFICATION OF INTERACTIONS

The practical or quantitative significance of toxic interactions at environmental levels of exposure is difficult to assess. As discussed in previous sections of this report and detailed further in Chapter 4, most published studies on interactions are not designed to quantify the magnitude of the interaction but focus primarily on qualitatively characterizing the nature of the interaction. In addition, quantitative measurements of interactions can only be made in reference to a non-interactive mathematical model, several of which are discussed in Chapter 4 and by NAS (1988a). Thus, the interpretation of the data in determining whether interactions occur can be highly model dependent. The available models also assume that the interaction among the compounds in the mixture is consistent over the entire range of relevant dose levels. An important consequence of this assumption is that the interaction is assumed to have no threshold. Few data are available for assessing the validity of this assumption.

The majority of studies that allow for any quantitative estimate of interaction involve acute exposures in which death or some other severe endpoint is measured. In such studies (Smyth et al., 1970; Hermens et al.,

1985a,b), interactions are expressed as the ratio of the observed LD_{50} to the expected LD_{50} based on the assumption of dose additivity. As discussed in Section 2.2., this is often referred to as the ratio of interaction. Most reported ratios of interaction do not exceed a factor of 2; the largest reported variation is a factor of 5 for an equivolume mixture of morpholine and toluene in the study by Smyth et al. (1970). Given the variations inherent in the conduct of bioassays, the significance of these variations from additivity is unclear. Few data are available regarding the variation of interactions among bioassays conducted by the same investigators (Sun and Johnson, 1960), and no interlaboratory studies have been conducted. Another source of uncertainty in assessing the implications of these ratios of interaction is that the nature and magnitude of interactions for severe toxic effects may not be the same as those for less severe effects. Furthermore, interactions that occur at high doses may not occur in the low-dose region. For example, the work of Plaa et al. (1982) on the well-documented potentiation of carbon tetrachloride-induced hepatotoxicity by acetone suggests that threshold concentrations exist below which an enhancement of toxicity may not occur. As discussed in Chapter 3, many of the biologic mechanisms by which interactions occur are also likely to be threshold phenomena.

As with acute bioassays of simple binary mixtures, very few studies on promotion or interaction were located that allow for the quantification of the interaction. One exception is the study summarized by Pfeiffer (1977) on interactions of carcinogenic and noncarcinogenic PAHs. This study, which involved 3000 mice, demonstrated both enhancement and inhibition of carcinogenic activity. Measured in terms of the observed proportion of responders versus the expected proportion of responders, variations from additivity ranged up to a factor of approximately 3. Most other studies

using experimental animals involve far less elaborate experimental designs: ethanol and vinyl chloride (Radike et al., 1981); cyclopenteno[cd]pyrene and benzo[a]pyrene (Cavalieri et al., 1983); and diethylnitrosamine and phenobarbitone or alcohol (Driver and McLean, 1986). These generally discuss or provide data that suggest variations from additivity, based on comparing the observed vs. the expected proportion of responders, by less than a factor of 10. Because observed response rates in most of these bioassays are over 10% and must be less than 100%, this observation may have more to do with the design and limitations of most bioassays than with the quantitative significance of interactions. No quantitative reviews of cocarcinogenic activity or promotion efficiency have been encountered in the literature that attempt a systematic and consistent analysis of the available but diverse animal data in order to estimate the significance and magnitude of these phenomena for risk assessment.

Epidemiologic studies on mixtures, as discussed in Section 2.2.2., focus on measuring relative risk associated with exposure to a complex mixture. Occasionally, interactions can be quantified between exposures to two complex mixtures or one complex mixture and another compound or agent. As with measurements of interactions from other types of studies, any quantitative estimate of interaction must be made with reference to a non-interactive model. For example, one of the most studied examples is the interaction between occupational exposure to asbestos fibers and cigarette smoking (Hammond and Selikoff, 1973; Hammond et al., 1979; Selikoff et al., 1968, 1980). In the study by Hammond et al. (1979), relative risks of about 5, 11, and 53 were noted for nonsmokers with occupational exposures to asbestos, smokers with no occupational exposure to asbestos, and smokers with occupational exposure to asbestos, respectively. As discussed in the mixture guidelines, this can be interpreted as evidence for a substantial

interaction (synergistic) between cigarette smoking and asbestos exposure if an additive risk model is assumed or as an indication of no interaction if a multiplicative risk model is assumed.

More recently, Steenland and Thun (1986) have reviewed the measurement of interactions in epidemiologic studies including a reappraisal of the data on cigarette smoking and exposures to asbestos, radon daughters, arsenic or chloromethyl ethers. As discussed by Steenland and Thun (1986), synergistic departures from an additive risk model have important public health consequences in that eliminating exposure to one agent can result in a greater reduction in risk than if no synergistic interaction occurred. The multiplicative risk model, on the other hand, is used in characterizing the etiology of a disease by determining if one risk factor modifies the effect of another risk factor. Of the epidemiologic studies reviewed by Steenland and Thun (1986), the Hammond et al. (1979) study showed the greatest deviation, by a factor of about 3.5, from risk additivity. Other deviations from risk additivity ranged from a factor of about 2 for smoking and radon or arsenic to 0.2 for smoking and chloromethyl ethers. In no instance did the observed relative risk for smoking and the other agent exceed the relative risk predicted by the multiplicative risk model. The recent reanalysis of the combined effects of cigarette smoking and exposure to radon daughters in the BIER IV report (NAS, 1988b) also noted evidence for a multiplicative or a "submultiplicative" model (i.e., the risk was greater than that predicted by the additive risk model but less than predicted by the multiplicative risk model) for uranium miners, although some support was found for a supra-multiplicative model.

3. AVAILABLE INFORMATION ON INTERACTION MECHANISMS

3.1. OVERVIEW

This chapter summarizes information on the chemical and biological mechanisms by which compounds interact. Such mechanisms include chemical-chemical interactions, pharmacokinetic effects and interactions at receptor sites and other critical cellular targets. For the most part, effects of different types (lethality, narcosis, enzyme induction, reproductive effects) or effects at different sites involve a common set of mechanisms. The phenomena of promotion and cocarcinogenicity have been extensively studied in a distinct body of literature and may involve a complex and as-yet-not-fully understood series of mechanisms, which are discussed at the end of this section.

As stated in the mixtures guidelines, toxicant interactions may be based on any of the processes that are significant to the toxicologic expression of a single compound: absorption, distribution, metabolism, excretion and activity at cellular site(s). In addition, compounds may interact chemically, causing a change in the biological effect or they may interact by causing different effects at different receptor sites. Of greatest practical importance is that most of these mechanisms are saturable as are most kinetic processes for single compounds. Consequently, many of the interactions observed at high doses may not be significant in the low-dose region.

Table 3-1, which summarizes these general modes of interaction along with some examples, was prepared using a modification of the basic scheme proposed by Veldstra (1956). As detailed in an extensive review by WHO (1981), "... the available evidence from in vitro and animal experiments and from human observations has shown that a limited number of mechanisms seem

TABLE 3-1

Chemical and Biological Bases of Toxicant Interactions
(See text for discussion, additional examples and references)

	Examples	
Bases of Interaction	Synergism or Potentiation	Antagonism
Chemical	formation of nitrosamines from nitrites and amines	dimethyl hydrazine reacts *in vivo* with pyridoxal phosphate (vitamin B6) to form a hydrazone, thus rapidly depleting tissue stores of this enzymatic cofactor (Cornish, 1969)
Biological		
Absorption	neurotoxicity of EPN (o-ethyl o-r-nitrophenyl phenylphosphorothioate) enhanced by aliphatic hexacarbons due in part to increased skin absorption (Abou-Donia et al., (1985)	dietary zinc inhibits lead toxicity in part by decreasing the percent dietary lead absorbed (Cerklewski and Forbes, 1976)
Distribution	increased lead levels in brain after treatment with dithiocarbamate/ thiuram derivatives (Oskarsson and Lind, 1985)	the mechanisms by which selenium protects against cadmium toxicity include decreasing the concentration of cadmium in liver and kidney and the redistribution of cadmium in the testes from the low-to-high molecular weight Cd-binding proteins (Chen et al., 1975)

TABLE 3-1 (cont.)

Bases of Interaction	Examples	
	Synergism or Potentiation	Antagonism
Excretion	decreased renal excretion of penicillin when co-administered with probenecid	arsenic antagonizes the effects of selenium in part by enhancing the biliary excretion of selenium (Levander and Argrett, 1969)
Metabolism	organophosphorous compounds (profenfos, sulprofos, DEF) potentiate the toxicity of fenvalerate and malathion by inhibiting esterase which detoxifies many pyrethroid insecticides (Gaughan et al., 1980)	selenium inhibits 2-acetylaminofluorene-induced hepatic damage and liver tumor incidence in part by shifting metabolism toward detoxification (ring hydroxylation) relative to metabolic activation (N-hydroxylation) (Marshall et al., 1979)
Interaction at Receptor Sites (Receptor Antagonism)	no information available	blocking of acetylcholine receptor sites by atropine after poisoning with organophosphates
Interaction Among Receptor Sites (Functional Antagonism)	no information available	interaction of histamine and norepinephrine on vasodilation and blood pressure
Interaction at DNA	no information available	Induction of DNA repair by exposure to alkylating agents

to account for the majority of the important known biological interactions." In other words, the basic mechanisms by which toxicants interact as detailed by Veldstra (1956) are based on classic pharmacologic principles that have not changed substantially over the past 30 years. While most of the best studied examples of the mechanisms of compound interactions are still from the pharmacologic literature on therapeutic drugs or abused substances such as ethanol (Seitz, 1985; Puurunen et al., 1983), an increasing number of examples are available showing similar mechanisms for compounds of occupational and environmental concern.

3.2. CHEMICAL INTERACTIONS

Many cases of direct chemical-chemical interactions lead to a decrease in toxicologic activity, and this is one of the common principles of antidotal treatment. Examples include the use of chelating agents to complex with metal ions, the inactivation of heparin by binding to protamine, and the use of ammonia orally as an antidote to the ingestion of formaldehyde through the formation of hexamethylenetetramine (Goldstein et al., 1974). This class of reactions has been referred to as chemical antagonism by Klaassen and Doull (1980).

Chemical reactions that lead to greater than additive effects have been less frequently documented. One example that has received considerable attention is the formation, in the stomach, of nitrosamines from nitrites and amines, which result in an increase in both toxic and carcinogenic effects (Weisburger and Williams, 1980; U.S. EPA, 1986a). Other examples include the formation of arsine and stibine from ores containing arsenic and antimony, respectively, which come into contact with strong acids in the stomach. Thus, while antagonism may be the most widely recognized result of

this mechanism for toxicant interaction in the classic pharmacologic literature, synergism or potentiation also occur and may be as significant in the environment.

3.3. PHARMACOKINETIC-BASED INTERACTIONS

Many examples of toxicant interactions are based on alterations in patterns of absorption, distribution, excretion or metabolism of one or more compounds in the mixture. Several reviews of these factors in the assessment of multiple chemical exposures are available (Anderson and Clewell, 1984; Plaa and Hewitt, 1981; WHO, 1981; Withey, 1981). All of these kinds of interactions essentially alter the bioavailability of the toxic agent(s) to the cellular site(s) without qualitatively affecting the toxicant-receptor site interaction.

3.3.1. Effects on Absorption. Most kinds of interactions based on alterations in absorption involve vehicle effects, the chemical formation of poorly absorbed conjugates or complexes, or decreases in gastrointestinal motility. Examples of such effects have been noted for oral and dermal exposures.

For example, the dermal toxicity of TCDD adsorbed on charcoal is considerably less than that of TCDD solubilized in a lipophilic medium. This is presumably due to the reduced availability of the charcoal-adsorbed TCDD for absorption by biological systems (Poiger and Schlutter, 1980). Conversely, dimethyl sulfoxide, a commonly used vehicle in dermal toxicity studies, is known to facilitate the absorption of many organic compounds across the skin, thus causing apparent potentiation when compared with less lipophilic vehicles (Goldstein et al., 1974). A similar mechanism appears to be involved in the enhancement of the neurotoxicity of o-ethyl-o-4-nitrophenyl phenylphosphonothioate by various aliphatic hydrocarbons when applied dermally to hens (Abou-Donia et al., 1985).

The acute oral toxicity of many compounds is substantially affected by the vehicle used, and many of these effects are probably due to differences in rate of absorption. For example, clioquinol administered orally is able to complex with many metals, facilitating their absorption, and has been implicated in an outbreak of heavy metal-induced subacute myelo-optic neuropathy in Japan (Tjalve, 1984). By contrast there are examples of compounds that form poorly absorbed complexes after oral administration such as tetracycline and calcium carbonate, as well as cholestyramine and cholesterol (Goldstein et al., 1974). Some compounds given orally, such as codeine, morphine, atropine and chloroquine, decrease the rate of gastric emptying, thus decreasing the rate of absorption of orally administered compounds. For the most part, such interactions usually lead to decreases in effects, because of the slower rate of absorption rather than increases in effects because of more complete absorption (Levine, 1973).

As discussed by Withey (1981) and confirmed in the literature reviewed for this report, there are no examples of toxicologically significant changes in absorption associated with the inhalation of mixtures. Murphy (1964) reported increased carboxyhemoglobin levels in mice and rats exposed to an ozone-CO mixture compared with CO alone. The exact mechanism of this response, however, has yet to be determined. Anderson and Clewell (1984), in their review of pharmacokinetic interactions and inhalation modeling, cite several examples of interactions based on effects on metabolism but none based on absorption. It has been hypothesized, however, that one mechanism by which particulates such as ferric oxide serve as respiratory cocarcinogens for benzo[a]pyrene (B[a]P) is by increasing residence time in the lung and, thus, allowing for more complete absorption of the compound. Alternatively, if absorbed on particles, the B[a]P is taken up by macrophages that have been shown to be capable of metabolizing B[a]P to a

proximate carcinogen. In addition it has been shown that cocarcinogenic particles facilitate uptake of the adsorbed chemical carcinogen across phospholipid bilayer membranes (Lakowitz and Hylden, 1978; Lakowitz et al., 1980).

3.3.2. Effects on Distribution. Distribution can play a role in compound interactions if a more active agent is displaced from an inactive site to a primary receptor site by a less active or inactive agent. One of the best documented examples of this kind of activity is the displacement of anticoagulants from plasma proteins by compounds such as barbiturates, analgesics, antibiotics or diuretics (Goldstein et al., 1974). Similarly, tri-o-tolyl phosphate decreases the binding of paraoxon to nonvital tissue in rat liver and plasma, consequently increasing the toxicity of paraoxon in rats (Lauwerys and Murphy, 1969). Since body fat represents a major nonvital storage site for many lipophilic xenobiotics, it may be anticipated that compounds that cause fat mobilization could result in similar potentiating effects (Withey, 1981).

Recently, Oskarsson and Lind (1985) demonstrated that dithiocarbamates and tetramethylthiuram disulfide may complex with lead and selectively increase the accumulation of lead in the brain. While the toxicologic significance of this interaction has not yet been demonstrated it can be reasonably presumed that this effect on distribution is likely to lead to a synergistic effect on the CNS effects of lead. A related mechanism was proposed by Larsson et al. (1976) for the teratogenic effect of maneb, which is antagonized by zinc acetate, suggesting that the teratogenic activity of maneb is due to the binding of zinc, causing embryonic zinc deficiency. Most examples cited above, however, result in greater than additive effects -- synergism or potentiation.

3.3.3. Effects on Excretion. Most examples of excretion as a basis for toxicant interaction involve compounds that are eliminated through the kidneys. For instance, probenecid or carinamide both competitively inhibit the elimination of penicillin, thus prolonging or potentiating its desirable therapeutic effect. Similarly, phenylbutazone inhibits the renal excretion of hydroxyhexamide, which can cause undesirably prolonged hypoglycemia. If a toxicant is eliminated through the kidneys, a stimulation of renal elimination can cause an antagonistic effect, as is seen with the coadministration of phenobarbital and sodium bicarbonate in which the increased urine alkalinity induced by the bicarbonate ion increases the excretion of phenobarbital (Goldstein et al., 1974).

A less direct effect on renal elimination has been suggested by Herschberg and Sierles (1983) for the substantial potentiation of the toxicity of lithium, which is eliminated through the kidneys, by indomethacin. These investigators suggest that the potentiation is due to the inhibition of prostaglandin synthesis by indomethacin, which in turn causes vasoconstriction and a decrease in the renal excretion of lithium.

As summarized by WHO (1981), several drugs and other chemicals are also able to compete for biliary excretion. Yamada et al. (1986) have demonstrated that quinidine has a marked inhibitory effect on the presystemic elimination of ajmaline by the liver when both compounds are administered concurrently to rats; similar observations have been noted in humans.

3.3.4. Effects on Metabolism. Altered patterns of compound metabolism have been shown to be the bases of many toxicant interactions (Anderson and Clewell, 1984; WHO, 1981). A major enzyme system involved in such interactions is liver microsomal mixed-function oxidases (MFO), which are involved in the activation or detoxification of a wide variety of compounds.

Both the types (e.g., different forms of cytochrome P-450) and levels of metabolic enzymes can be induced by agents such as phenobarbital, and enzyme activity can be inhibited by agents such as piperonyl butoxide (Goldstein et al., 1974). Thus, depending on whether or not the toxicant is activated or detoxified, inducers or inhibitors of this enzyme system may cause synergistic/potentiating effects or antagonistic effects (Freeman and Hayes, 1985; Leonard et al., 1985). Toxicant interactions involving the MFO can be complex and depend on both dose and duration of exposure, with some compounds causing an initial inhibition of enzyme activity followed by a marked induction of activity (WHO, 1981). Although liver microsomal MFO are the most commonly studied enzymes involved in toxicant interactions, MFO in other tissues may also play an important role in toxicant interactions as may other enzyme systems, such as alcohol and aldehyde dehydrogenases, monamine and diamine oxidases, dehydrochlorinases, azo and nitro reductases, hydrolases and enzyme systems involved in conjugation reactions. For instance, ethanol serves as an antagonist of the toxic effects of methanol by acting as a competitive inhibitor of alcohol dehydrogenase, thus suppressing the formation of formaldehyde and formic acid from methanol (Goldstein et al., 1974).

3.3.5. Interactions at Receptor Sites or Critical Cellular Targets. All of the biological modes of toxicant interactions discussed above -- absorption, distribution, excretion and metabolism -- are essentially dispositional, affecting the amount(s) of toxicant(s) reaching the primary receptor(s). Most of these kinds of interactions can involve either synergism/potentiation or antagonism. Another biological basis for toxicant interactions involves events that occur at cellular receptor sites or among receptor sites and are usually thought to result solely in antagonistic

interations. The antagonistic nature of interactions that occur at the same receptor site was discussed by Veldstra (1956):

> ...we may say that the effect of a combined action of two compounds at the same site of primary action will not result in a synergism, but will, generally, even be unfavorable. The competition for the receptor will usually decrease the frequency of the best interactions, and with decreasing intrinsic activity of one of the components the combined action will more and more take the form of a competitive antagonism.

Examples of such interaction include the antagonistic effects of oxygen on carbon monoxide, atropine on cholinesterase inhibitors and naloxone on morphine (Goldstein et al., 1974). The antagonistic consequences of this kind of toxicant interaction are so consistent that it has been termed "receptor antagonism" by Klaassen and Doull (1980) and "pharmacological antagonism" by Levine (1973). While it seems conceivable that one compound could increase the intrinsic activity of another compound by modifying the receptor site -- analogous to the effect of modulators on regulatory enzymes -- such interactions have not been demonstrated.

Interactions of agents among receptor sites are also thought to result primarily in antagonistic effects and has been referred to as "functional antagonism" by both Klaassen and Doull (1980) and Levine (1973). This kind of interaction is most commonly defined as two or more compounds acting on different receptor sites and causing opposite effects on the same physiological function. Examples include the opposite effects of histidine and norepinephrine on vasodilation and blood pressure and the anticonvulsive effects of barbiturates on many compounds that cause convulsions. Nevertheless, that interactions among receptor sites uniformly result in an antagonistic response is not certain, particularly when the receptor sites act on

different physiological systems. The rationale for this statement was presented by Veldstra (1956):

> The sites of action for two compounds having the same type of activity may be different. This is the case when the effect can be caused either by a direct stimulation or by the annihilation of an inhibition. In both cases, the combination of two compounds, linked in parallel or in series, as it were, may well result in a synergistic effect. When the components of a combination possess different sites of action and different types of activity, no plausible prediction about the possibility of synergism can be made, unless their mode of action is well known.

While examples of such interactions have not been well characterized in the literature, the potentiation of carbon tetrachloride by chlordecone may be at least partly mediated by an inhibition of hepatocellular repair (Lockard et al., 1983).

Another possible illustration of Veldstra's argument is presented in the work of Alstott et al. (1973), who examined the acute lethal effects of combinations of 1-methylxanthine and ethanol on mice, and noted two basic kinds of effects: kidney dysfunction and increased respiratory rate and depth. In animals exposed to mixtures in which the ratio of 1-methylxanthine to ethanol was relatively high, antagonism of acute lethal toxicity was observed; however, in mixtures in which the same ratio was relatively low, a synergism of acute lethal toxicity was observed. This indicates that in cases where toxicants interact at more than one cellular site, the nature of the interaction can be either antagonistic or synergistic. The complicating factor of the "asymmetric" pattern of interaction observed by Alstott et al. (1973) is discussed in greater detail in Chapter 4.

3.3.6. Promotion and Cocarcinogenicity. Mechanistic studies on promotion and cocarcinogenicity have been active areas of research over the past decade. The extensive literature has been the subject of several comprehensive general reviews (Slaga, 1984; Williams, 1984; Yamasaki, 1984) as well

as reviews that have focussed on specific topics such as hepatocarcinogenesis (Pitot et al., 1982; Schulte-Hermann, 1985), the inhibition of cellular communication by promoters (Trosko et al., 1983), the induction of superoxide anions by promoters (Troll and Wiesner, 1985), and the binding of promoters to protein kinase C in cell membranes (Hecker, 1985).

Various investigators have used different but generally overlapping mechanistic schemes to categorize the types of information on promotion and cocarcinogenicity. Table 3-2 summarizes the mechanisms based on the approach taken by Williams (1984), who also provides many specific examples. As mentioned earlier in this chapter, one possible mechanism of cocarcinogenesis is to increase cellular exposure to an initiating substance. Particulate ferric oxide can serve as an effective vehicle for delivery of an adsorbed carcinogen, such as benzo[a]pyrene to the target organ, namely lung. The particles are subject to phagocytosis by pulmonary alveolar macrophages, which can elute the benzo[a]pyrene (Autrup et al., 1979), transport the compound to a distant site or metabolize it. Likewise solvents may also serve as cocarcinogens by increasing efficiency of carcinogen delivery.

Agents may serve as cocarcinogens by affecting the metabolism of a procarcinogen such that a more active metabolite or that a greater quantity of reactive metabolites is maintained in the cell. This can be accomplished by induction of metabolic enzyme systems as described previously or by depletion of or competition with detoxification systems. An example of a compound with this latter activity is diethyl maleate, which is known to deplete liver of glutathione, a cellular nucleophile. Depletion of glutathione increases hepatotoxicity (and presumably the potential for hepatocarcinogenicity) of aflatoxin B1 (MgBodile et al., 1975).

TABLE 3-2

Mechanisms of Promotion and Co-carcinogenicity*

Co-carcinogenesis:

1. Increased uptake of carcinogen
2. Increased proportion of carcinogen activation
3. Depletion of competing nucleophiles
4. Inhibition of the rate or fidelity of DNA repair
5. Enhancement of the conversion of DNA lesions to permanent alterations

Promotion:

1. Enhancement of expression of neoplastic phenotype

   Inhibition of differentiation

2. Stimulation of cell proliferation

   Cytotoxicity
   Hormone Effects

3. Cell membrane effects

   Induction of proteases
   Inhibition of intercellular communication

4. Immunosuppression

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*Source: Williams, 1984

Another possible mechanism of cocarcinogenesis takes place at the level of DNA damage. It is known that certain compounds can act as co-mutagens in *in vitro* systems: norharman for aniline and benzo[e]pyrene for benzo[a]-pyrene. These interactions could take place at any of a number of steps in the mutagenic process, including enhancement of mutagenic metabolite production. It is known, however, that DNA that is being actively transcribed is more susceptible to damage than is "resting" DNA. It seems plausible then that some agents could enhance initiation by making the DNA more susceptible to damage, for example, by holding it in a single stranded configuration, or by increasing transcription.

Interference with error-free DNA repair is another way in which a cocarcinogen could work. Induction of an error-prone repair system by DNA-damaging agents is a well-documented phenomenon in *Escherichia coli*. In mammalian cells, certain systems, such as that responsible for repair of alkylation damage, also appear to be inducible (Swenberg et al., 1982). There is, however, no evidence as yet of an error-prone repair system that could be turned on by either a DNA-damaging or a co-mutagenic agent. It has been reported that some agents reduce the rate of DNA synthesis, including repair synthesis. Such a reduction in rate of repair could have the effect of increasing the number of permanent DNA alterations or mutations (Williams, 1984). It has been reported that a compound, 3-aminobenzene, which inhibits the activity of the poly(ADP-ribose) polymerase specific to DNA repair enhances the formation of liver foci initiated by another compound (Takahaski et al., 1982).

The classic two-stage initiation-promotion sequence proposed by early investigators (Berenblum, 1941a,b) is more likely to reflect experimental design constraints than two simple discrete mechanistic stages. Slaga (1984) has described two separate stages in promotion in which the initiated

cell develops to a benign tumor, as well as two stages of progression. The first stage of progression is that in which a benign tumor develops into a malignant tumor, while in the second stage the malignant tumor metastasizes; each of these stages may involve different mechanisms of interactions.

As mentioned in Chapter 2, the practical significance of the distinction between tumor initiation and tumor promotion is that the former is commonly regarded as having no threshold while the later is often thought to display a threshold below which no tumor promotion will occur (Driver and McLean, 1986). This view, however, has been challenged by Yamasaki (1984), who claimed that the data are not adequate to determine if promotion evidences a true dose-threshold. Rather, it was suggested that because at least some stages of promotion are reversible, promoters display a dose-schedule threshold (i.e., the dosing schedule is of greater importance than the total administered dose) that is different from that of initators or complete carcinogens.

The implications of mechanisms of promotion for risk assessment are further complicated by the fact that some compounds can interact with promoters to increase or diminish promoting efficiency (Schulte-Hermann, 1985; Slaga, 1984; Williams, 1984). For example, Sleight (1985) has reported that 3,3',4,4',5,5'-hexabromobiphenyl enhances the promoting efficiency of 2,2',4,4',5,5'-hexabromobiphenyl and that this may explain why commercial mixtures of polybrominated biphenyls have a greater promoting ability than any of the individual congeners.

3.3.7. Interactions and Developmental Toxicity. Developmental toxicity is indicated by many different types of endpoints including death, structural abnormality, altered growth and functional deficits (U.S. EPA, 1986b).

These various endpoints are likely to arise as a consequence of any of a number of cellular processes including mutations, membrane changes, changes in gene expression, or other events leading to cell death. There is potential for interaction to occur at any of these processes that would be manifested as increases or reduction in developmental measurements. It is generally assumed that there are dose thresholds for developmental effects based on the rationale that the embryo has some capacity for repair of damage or replacement of dead cells. Interactions could have the effect of raising or lowering this threshold as for other systemic effects.

4. MATHEMATICAL MODELS AND STATISTICAL TECHNIQUES

4.1. INTRODUCTION

This chapter presents a review and evaluation of some representative statistical methods for the assessment of toxic responses to mixtures. There are four different classes of methods, described as follows: dose addition, response addition, generalized linear models and response surface models. The theoretical framework for each class is discussed, and variations within each class are described. Some recently proposed methods for use in analysis of mixtures data are also presented, along with an evaluation of applications of statistical methods in the mixtures literature.

Interaction is defined statistically as the effect of two or more treatments applied jointly that cannot be predicted from the average responses of the separate factors. This concept of dependence of the effect of one factor on the level of another factor is a fundamental scientific idea. When interaction is present, the result of two or more factors applied jointly may result in either positive or negative deviations from the expected result for each factor taken one at a time. As noted in Chapter 1 and Appendix A, when a large positive deviation is present, the common biological terminology used is synergism. When a negative deviation is present, antagonism is said to be present. In the special case where a deviation occurs when the two factors are applied together, but one factor by itself has no effect, the positive deviation is called potentiation, and the negative deviation is called inhibition.

The above definitions are contingent on how the expected (or "no-interaction") effects are defined (Berenbaum, 1985a). There are two general classes of models for joint action that assume no interaction. These classes describe either dose addition or response addition.

4.2. DOSE ADDITION

Dose addition, or simple similar action (Finney, 1971), assumes that the compounds in a mixture act as if they are dilutions or concentrations of each other. For example, in a binary mixture, a dose containing z_1 units of compound 1 and z_2 units of compound 2 would, under dose addition, behave exactly as a dose of (z_1+pz_2) units of compound 1 alone, where p is the potency of compound 2 relative to compound 1. In particular, assume the two compounds have parallel regression lines of probits on log doses as follows:

$$Y_1 = \alpha_1 + \beta \log Z \qquad (4\text{-}1)$$

$$Y_2 = \alpha_2 + \beta \log Z \qquad (4\text{-}2)$$

where Z is the dose. Simple similar action is said to occur when the response to a mixture containing amounts Z_1 and Z_2 units of compounds 1 and 2, respectively, has a response probit of the form

$$Y = \alpha_1 + \beta \log(Z_1+pZ_2) \qquad (4\text{-}3)$$

Alternatively, if the mixture is a total dose Z of the two compounds in proportions f_1 and f_2 then the mixture has a response probit of the form:

$$Y = \alpha_1 + \beta \log(f_1+pf_2) + \beta \log Z \qquad (4\text{-}4)$$

Note that the assumption of parallelism is implicit in the formulation of this model (4-1 and 4-2).

One method for testing for dose additivity is to assess the adequacy of fit of the model (4-4) rewritten as

$$Y = \alpha_3 + \beta \log Z \qquad (4\text{-}5)$$

Alternatively, when several mixtures of different proportionate concentrations are to be tested, several different estimates of p can be obtained.

The sum of squares between observed and predicted response from equation 4-4 can then be minimized with respect to α, β and p, and an overall estimate of p is found. Testing for dose additivity is then done by comparing this sum of squares against one where values of p were estimated separately for each dose series.

Finney (1971) has also proposed the following model to be used for assessment of interaction:

$$Y = \alpha + \beta \log(f_1+f_2p + K(f_1f_2p)^{0.5}) + \beta \log Z \qquad (4\text{-}6)$$

where α, β, p are as defined before, Z is the sum of Z_1 and Z_2, and K is the coefficient of interaction. A positive value of K indicates synergism; a zero value, simple additivity; and a negative value, antagonism.

This model assumes a constant interaction throughout the entire range of proportions of individual components. In order to allow for a less restrictive assumption, Durkin (1981) made the following modification:

$$Y = \alpha + \beta \log(f_1+f_2p+(K_1f_1+K_2f_2)(f_1f_2p)^{0.5}) + \beta \log Z \qquad (4\text{-}7)$$

The properties of this model, however, have yet to be critically evaluated. Durkin (1981) also proposes several statistical methods for testing for departures from simple additivity. For example, the model for symmetrical interactive action (Finney's model from equation 4-6):

$$(1/Z_3) = (1/Z_1)[f_1+f_2p+K(f_1f_2p)^{0.5}] \qquad (4\text{-}8)$$

where Z_3 is the observed LC_{50} for the mixture and Z_1 is the LC_{50} for compound 1, can be fit using weighted linear regression analysis. Similarly, the model for asymmetrical interactive action (Durkin's model from equation 4-7),

$$(1/Z_3) = (1/Z_1)[f_1+f_2p+(K_1f_1+K_2f_2)(f_1f_2p)^{0.5}] \qquad (4\text{-}9)$$

can also be fit by similar means. The hypothesis that

$$K(p^{0.5})/Z_1 = 0 \qquad (4\text{-}10)$$

or

$$(K_1f_1+K_2f_2)(p^{0.5})/Z_1 = 0 \qquad (4\text{-}11)$$

can then be tested. If the relevant hypothesis is not rejected, then the data are consistent with simple additivity. Let SSE_1 denote the sum of squares error for the simple additivity model, and SSE_2 the sum of squares error for the relevant interactive model. Define then

$$F = S_3/S_2$$

where: $S_3 = (SSE_1 - SSE_2)/(M - g)$

$$S_2 = SSE_2/(n - (M + 1))$$

where n is the number of measurements, g is the number of parameters in the model for simple similar action and M is the number of parameters in the interactive model. Thus, this F statistic has M-g and n-(M+1) degrees of freedom. Again, the properties of this method have not been rigorously evaluated.

Another method proposed for testing for simple additivity is to divide the observed LC_{50} of the mixture by the LC_{50} predicted from simple additivity (Durkin, 1981). This method is to be used when LC_{50} estimates of components and mixtures vary substantially, especially those from experiments conducted at different times, thus obscuring trends to nonlinearity. Under the null hypothesis of simple similar action the observed LC_{50} of the mixture will equal the LC_{50} predicted from equation 4-5. Explicitly

$$\frac{Z_{3obs}}{Z_{3pred}} = \phi_1 + \phi_2 = 1 \qquad (4\text{-}12)$$

where ϕ_1 and ϕ_2 can be considered the proportions of the mixture toxicity attributable to compounds 1 and 2, respectively. Under a

hypothesis of interaction such as given by equations 4-8 or 4-9, then

$$\frac{Z_{3obs}}{Z_{3pred}} = \phi_1+\phi_2 + K(\phi_1\phi_2)^{0.5} \qquad (4\text{-}13)$$

or

$$\frac{Z_{3obs}}{Z_{3pred}} = \phi_1+\phi_2 + K_1\phi_1\ (\phi_1\phi_2)^{0.5} + K_2\phi_2\ (\phi_1\phi_2)^{0.5} \qquad (4\text{-}14)$$

This heuristic method does not have set rules for determination of statistical significance and the method has not been rigorously evaluated. Several variations on this approach have been discussed in Section 2.4.

The dose addition model can be extended beyond two substances. The mathematics of such models, however, are even more complicated, and data requirements for fitting these models increase substantially as well. Therefore, using current information, these models can be of practical use only with mixtures of relatively few component chemicals.

Plackett and Hewlett (1952) criticize the dose addition model on two points. First, the parameter K is inadmissible for certain values of Z_1 and Z_2. Second, this model assumes that p, the potency of compound 2 relative to compound 1, is fixed and constant for all organisms under study, a condition that they feel is unnecessarily restrictive.

4.3. RESPONSE ADDITION

Response addition models were first proposed by Bliss (1939). In the original representation for "independent joint action" of the two chemicals, Bliss (1939) assumed that the two chemicals acted on different physiologic systems. This assumption can be generalized to functional independence of the two separate effects, even if they are on the same organ system. Define the following:

D_1 = dose of chemical 1

P_1 = proportion of animals responding to D_1

and similarly for chemical 2. Bliss (1939) noted that the proportion of animals that respond to the mixture depends not only on P_1 and P_2 but also on the correlation between the two distributions of individual tolerances to the chemicals. If there is parallelism in the susceptibility to the two chemicals so that the correlation is 1, i.e., if the ordering of the animal sensitivities to chemical 1 is the same as the ordering for chemical 2, then the most toxic chemical will elicit the response first. The mixture response is then:

$$P = \max(P_1, P_2) \tag{4-15}$$

As noted in the U.S. EPA (1986a) mixture guidelines, if the tolerance correlation is -1, i.e., if the animal least sensitive to chemical 1 is most sensitive to chemical 2, and so on throughout the range of sensitivity, then

$$P = \min(P_1+P_2, 1) \tag{4-16}$$

For $P<1$, equation 4-16 is the simplest response addition formula:

$$P = P_1 + P_2 \tag{4-17}$$

Other tolerance correlations give P values between these extremes. If the correlation is 0, then one obtains the familiar model for statistical independence:

$$P = P_1 + P_2 - (P_1P_2) \qquad (4\text{-}18)$$

Hewlett and Plackett (1964) discuss a different class of models based on combining responses of two chemicals. Instead of counting the number of animals responding, they model the number of tissue receptors that are affected by the chemicals. Their fundamental assumption is that the tissue damage can be described by chemical complexing of the tissue receptor with the administered chemical. The manner of competition of chemical molecules for these tissue receptors is assumed to be described by laws of mass action, so that key model parameters are the chemicals' dissociation constants for complexes. Their model also assumes that a quantal response occurs only when an underlying graded response, E, exceeds some critical threshold, E_c. The model assumes that the joint action of two compounds is the result of competition for the same set of receptors. Using Hewlett and Plackett's (1964) notation, let m_1 and m_2 be the reciprocals of the dissociation of the receptor-compound complexes for compounds 1 and 2, respectively, and let ω_1 amd ω_2 be the respective molar concentrations of these compounds at their respective sites of action. The graded response to compounds 1 and 2 is

$$E(\omega_1,\omega_2) = (n_1\omega_1 + n_2\omega_2)/(1 + m_1\omega_1 + m_2\omega_2) \qquad (4\text{-}19)$$

where

$$n_i = A_i m_i [r], \; i = 1,2,$$

A_i is the intrinsic activity of compound i, and [r] is the total molar concentration of receptors. Let ω_i' denote the action tolerance (or threshold) for compound i, defined as the amount of compound which, when acting alone, is just insufficient to produce the quantal response in the individual. A quantal response will then occur when $\omega_i > \omega_i'$.

When compound 1 is an agonist and compound 2 is an antagonist (Case 1), quantal response occurs when

$$\omega_1/(1 + m_2\omega_2) > \omega_1' \tag{4-20}$$

If ω_1' is log-normally distributed in the population of individuals considered then the model for the normal equivalent deviate of response is

$$Y = \varepsilon + \theta' \log (\omega_1/(1 + m_2\omega_2)) \tag{4-21}$$

If the relation between the acting concentration of compound i, ω_i, and the administered amount, Z_i, is assumed to be

$$\omega_i = \mu_i Z_i^{\eta_i} \tag{4-22}$$

then equation 4-21 becomes

$$Y = \alpha_1 + \beta_1 \log(Z_1/(1 + \psi Z_2^{\eta_2})) \tag{4-23}$$

If both compounds are agonists both elicit the same maximum response (Case 2) then quantal response occurs when

$$(n_1\omega_1 + n_2\omega_2)/(1 + m_1\omega_1 + m_2\omega_2) > K \tag{4-24}$$

where K is the critical graded response for compound 1 alone and for compound 2 alone. Thus quantal response occurs when

$$\omega_1/\omega_1' + \omega_2/\omega_2' > 1 \tag{4-25}$$

Let $\delta_i = \omega_i/\omega_i'$. Then the nonresponse proportion is

$$q = Pr(\delta_1+\delta_2 \leq 1) \qquad (4\text{-}26)$$

which can be evaluated if ω_1' and ω_2' have a bivariate normal distribution. If the maximum response attainable by compound 1 is greater than that attainable by compound 2 (Case 3), then quantal response occurs if

$$(n_1\omega_1 + n_2\omega_2)/(1 + m_1\omega_1 + m_2\omega_2) > n_1\omega_1'/(1 + m_1\omega_1')r \qquad (4\text{-}27)$$

As $n_2 \to 0$, Case 1 results. As $(n_2/m_2) \to (n_1/m_1)$, Case 2 results. Otherwise, subjecting this model to the same derivations as for Case 1 results in "a model which has doubtful practical value on account of the number of parameters involved" (Hewlett and Plackett, 1964).

The parameter q is evaluated by integrating the bivariate normal density function over the appropriate region. Subsequent analysis of dose-mortality data then uses the log-dose-probit response line, which is curvilinear under independent action of the two compounds and is skewed upward as response increases and the correlation coefficient between the action tolerances for the two compounds decreases. Bliss (1939) notes that curvilinearity of a dose-response curve is difficult to test in experimental data, and Durkin (1981) attributes the paucity of studies with examples of response addition to this difficulty.

Similar to the dose addition models, the response addition models can be easily generalized to more than two chemicals. The complexity of such models and the accompanying extreme data requirements, however, make such models of little practical use.

4.4. GENERALIZED LINEAR MODELS

For the ordinary linear model, "interaction" is taken by statisticians to mean a departure from response additivity assuming a normal distribution of the response variable. For generalized linear models (e.g., logistic, log-linear, log-probit and multistage models), interaction is taken to mean a departure from additivity for a transformation of the response variable. For instance, in the log-linear model

$$\log(p_{ij}) = m + a_i + b_j + d_{ij},\ i=0,1,\ j=0,1 \qquad (4\text{-}28)$$

where $a_0=b_0=d_{00}=d_{10}=d_{01}=0$, and where p_{ij} denotes the proportion responding in the group receiving dose level i of compound 1 and dose level j of compound 2, the d_{11} term describes the presence and extent of interaction between compounds 1 and 2. Similarly, in the logistic model

$$\log(p_{ij}/(1-p_{ij}) = m + a_i + b_j + d_{ij},\ i=0,1,\ j=0,1 \qquad (4\text{-}29)$$

where p_{ij} is as before, $a_0=b_0=d_{00}=d_{10}=d_{01}=0$, and d_{11} describes the interaction.

Although the examples of generalized linear models given above are applicable only to experiments with simple binary mixtures, these models can be extended to experimentation with three or more compounds. The difficulty in doing so is not in the mathematics, but rather time and expense incurred in the conduct of appropriately designed factorial experiments.

Use of fractional factorial designs can be used more economically, but still can be lengthy if whole animal lifetime studies are conducted.

Moreover, fractional designs also assume that one or more higher order interactions are zero, when information on all interactions may be the object of the exercise.

Nonlinear terms can also be incorporated into generalized linear models and the Box-Tidwell fitting technique can be applied to obtain parameter estimates (McCullagh and Nelder, 1983). In particular, if $g(X;\theta)$ is the covariate of interest where θ is unknown, the expansion of $g(X;0)$ about an initial value θ_0 is obtained to derive the linear approximation

$$g(X;\theta) \sim g(X;\theta_0) + (\theta-\theta_0)[\partial g/\partial\theta]_{\theta=\theta_0} \qquad (4\text{-}30)$$

Therefore, if the model contains a nonlinear term of the form

$$\beta g(X;\theta)$$

then replace it by two linear terms of the form

$$\beta u + \gamma v$$

where $u = g(X;\theta_0)$

$v = [\partial g/\partial\theta]_{\theta=\theta_0}$

$\gamma = \beta(\theta-\theta_0)$

The estimation procedure for θ is then iterative as follows:

1. Fit the generalized linear model with covariates u and v
2. Obtain $\theta_1 = \theta_0+\gamma/\beta$ as the improved estimate
3. Iterate to convergence

McCullagh and Nelder (1983) noted that this technique is highly useful and probably under-used, but cautioned that this method is not appropriate for the inclusion of many nonlinear terms since the estimates of these parameters will have large sample variances and will usually be highly correlated with the linear parameters and possibly with each other.

Elashoff et al. (1987) describe a modification of the proportional hazards model to allow for the incorporation of competing risk for death to evaluate interactions between two chemicals in a 2x2 carcinogenicity experiment. For the analysis of tumor incidence data, they test for interaction using the additivity index (Wahrendorf et al., 1981) as follows:

$$I = \log(q_{10}/q_{00}) + \log(q_{01}/q_{00}) - \log(q_{11}/q_{00}) \qquad (4\text{-}31)$$

where q_{00} is the background probability of not developing a tumor, q_{10} and q_{01} are the probabilities of developing a tumor when compounds 1 and 2 are administered alone, respectively, and q_{11} is the probability of not developing a tumor when compounds 1 and 2 are administered concurrently. If $I>0$, synergy is said to have occurred, and if $I<0$ then antagonism is said to have occurred.

The time-to-death data is important to consider in addition to the tumor incidence data when lethal nontumorigenic toxicity in the doubly exposed group relative to the singly exposed groups is excessive since it can cause a negative bias in I. Therefore, they used the proportional hazard model

$$\Pr(\text{survival without tumor at T years for treatment ij}) = \exp[-\int_0^T (h'_{ij}(t) + h_{00}(t))\, dt], \qquad (4\text{-}32)$$

where h' represents the incremental force of mortality due to treatment. To test for interaction they use the null model

$$h'_{10}(t) + h'_{01}(t) - h'_{11}(t) = 0 \qquad (4\text{-}33)$$

A test statistic developed by Korn and Liu (1983), which uses a Mantel-Haenszel approach, is then used to test for no interaction with respect to time to death.

Generalized linear models have also been proposed for multi-effect data on the complete mixture. The responses are graded (nonquantal) and the overall toxicity of the mixture is assigned to a severity category (Hertzberg, 1987). A link function transforms the response frequencies for each dose in each severity category, and the transformed response for these ordered categories is then regressed on a linear function of dose (duration could also be included as a covariate). For example, if effects are categorized as "none," "mild," "moderate" and "severe," and if "mild" effects were considered tolerable, then one could determine the risk of "moderate or severe" effects for a given mixture dose. The model is similar to those discussed previously. For example, for the logistic link function, the counterpart to equation 4-29 is

$$\log[G_j/(1-G_j)] = T_j - b^*[\ \log(D) - \overline{\log(D)}\ ] \qquad (4\text{-}34)$$

where D now denotes the dose of the complete mixture, the overbar denotes the mean of log(D), and j denotes the severity category. The response variable G is a function of the mixture dose D and represents the cumulative response frequency at dose D, i.e., the organisms responding at severity level j or less. If P_k is the proportion of animals responding to dose D at severity level k, then the transformed response is

$$G_j = \sum_{k=1}^{j} P_k \qquad (4\text{-}35)$$

The probabilistic risk estimate from such a model is obtained by inverting the link function, to give the risk of an effect worse than category j,

$$p(J>j) = 1 - \exp[F_j(D)] / (1 + \exp[F_j(D)]) \qquad (4\text{-}36)$$

where F represents the right-hand side of equation 4-34.

A mixture of chemicals is likely to induce several different kinds of effects in different organs. Applying the previously discussed response models to each kind of effect, even if data were available on the complete mixture, would generate several dose-response curves, and would require some statistical combination algorithm to address the multiplicity of effects. The recasting of the risk problem using severity categories is mathematically simpler, and also avoids the difficult issue of correlation of specific toxic effects across species. The risk assessor then evaluates only the risk of general systemic toxicity, e.g., the risk of unacceptable effects. This procedure also allows the toxicologist to assign multiple effects to a higher severity category. For example, "mild" effects in several diverse organs and tissues would be deemed "moderate" and unacceptable when considered as a composite toxic response.

4.5. RESPONSE SURFACE MODELS

When a 2x2 factorial design is used to study the interaction of two compounds, no information is gained about how the response changes with changes in the magnitude of exposure to both compounds 1 and 2. If the dose ranges for these compounds are more completely studied, however, the economic requirements increase as well. For instance, with only three nonzero doses of each compound in a binary mixture, 16 treatment groups must be studied if all possible combinations of the two compounds are used. An alternative

approach is motivated by conceptualizing the response to the joint exposure as forming a surface over the experimental plane with peaks and valleys. Designs that maximize or minimize this surface by sequential exploration are called response surface models. They are most frequently used in industrial experimentation where the response can be measured quickly and where a small number of factors are to be combined. Thus, their utility for the study of mixtures of even moderate complexity or for use in long-term toxicity studies is questionable.

4.6. SUMMARY OF INTERACTION DATA BASE

A survey of the statistical methods utilized in studies pertaining to mixtures was conducted using those papers included in the U.S. EPA interaction data base (U.S. EPA, 1988) as well as papers retrieved subsequent to the construction of the data base. A total of 462 relevant references were included in this survey, which also examined the type of mixture studied (binary, simple, or complex), whether the study was descriptive or mechanistic in its approach, and whether the mixture included carcinogenic compounds. A relevant reference was considered one in which both methods and data were presented, i.e., abstracts and reviews were not included. Of the 331 references contained in the interaction data base, 307 were considered relevant. An additional 155 studies were also included in this survey (Table 4-1).

A summary of the types of studies examined and the statistics used in each is presented in Table 4-1. Individual columns are used for those papers found in the data base and those not included so that an exclusive analysis of the data base can be made separately. The first group of categories pertains to the general characteristics of each individual study.

TABLE 4-1

Survey of Interaction Studies Methodologies*

	Data Base	Other	Total	Percent (%)
Number of studies	307	155	462	--
Nature of Individual Studies				
Binary mixture	294	150	444	96.1
Simple mixture	24	16	40	8.7
Complex mixture	17	7	24	5.2
Descriptive	276	136	412	89.2
Mechanistic	61	50	111	24.0
Noncarcinogen	261	118	379	82.0
Carcinogen	46	37	83	18.0
Statistical Breakdown of Individual Studies				
Student's t-Test	85	52	158	34.2
No Statistics	85	35	119	25.8
Statistics Not Specified	71	35	106	22.9
Analysis of Variance	34	16	52	11.3
Chi-Square Test	17	12	29	6.3
Neumann-Keuls Test	13	3	16	3.5
Mann-Whitney U Test	7	5	12	2.6
Wilcoxon Test	3	7	10	2.2
Duncan's Multiple Range Test	9	1	10	2.2
Fisher Exact Test	4	4	8	1.7
Tukey's Test	3	3	6	1.3
Dunnet's Test	3	2	5	1.1
Kruskall-Wallis Test	3	2	5	1.1
Least Significant Difference	3	1	4	0.9
Finney Additivity Formula	1	3	4	0.9
F Test	2	1	3	0.6
Scheffe's Test	1	2	3	0.6
2-Sample Rank Test	1	0	1	0.2
Fisher-Yates Test	0	1	1	0.2
Mantel-Haenszel Procedure	0	1	1	0.2

*Refer to text for explanation of individual categories.

Interactions result from a binary mixture (two constituents), a simple mixture (more than two but less than dozens of identifiable components), or a complex mixture (dozens or more constituents, many of which are unidentified or present in low concentration). Several studies used more than one type of mixture involving, in most cases, the effect of one compound on the interaction of two other compounds. In other instances, the interaction between two single components, e.g., carbon tetrachloride and phenobarbital (binary mixture), as well as the interaction between a single compound and a mixture of compounds, e.g., carbon tetrachloride and PCBs (complex mixture), would be investigated in the same study. The total number of evaluations is then much larger than the number of references, although it is obvious that an overwhelming number of evaluations pertain only to binary mixtures.

These studies are also segregated as to whether they analyze an interaction mechanistically, descriptively or both. A descriptive study is one that only looks at one or more toxic endpoint(s) to characterize the magnitude of the interaction without examining the underlying cause(s) for the interaction. Such endpoints commonly include LD_{50} values, serum enzyme levels, and sleeping times. Mechanistic studies, on the other hand, attempt to quantify changes in the absorption, distribution, metabolism, excretion, receptor binding, or physical characteristics of a compound. Examples of mechanistic endpoints include urinary metabolite profile, intestinal absorption, hepatic enzyme activities, and tissue distribution. Several studies incorporate both approaches by attempting to correlate a change in toxicity with the biological or chemical bases of the interaction. For example, several studies have examined the effects of certain enzyme inducers such as phenobarbital, 3-methylcholanthrene, or PCBs with a change

in hepatotoxicity induced by carbon tetrachloride. Table 4-1 indicates that 61 studies (412 descriptive + 111 mechanistic - 462 total) utilized both strategies.

Finally, the number of studies involving carcinogenic endpoints was determined. A carcinogen study is defined as one in which a determination of tumor frequency, latency or incidence is made. Studies in which known carcinogens were used but were not of sufficient duration for tumor formation were included in the noncarcinogen category. Unlike the other categorizations, a study was classified as either carcinogen or noncarcinogen but not both.

The use of statistical methods as specifically stated in either the methods section, in tables or figures, or in the text was tabulated for each study. As reflected in Table 4-1, the most widely used procedure is the Student t-test, which was utilized in over one-third of the studies. This test was frequently used in conjunction with other methods such as analysis of variance (ANOVA). Most often, however, the t-test was the only method employed. A noteworthy finding was that one-quarter of the studies in the survey contained no reference to any statistical procedures. In addition, nearly 23% of the studies did not specify the type of statistical tests used. In these cases, either the p values were given in the text or in the footnotes to tables or figures without explanation or the use of statistics was referenced to another source. In one study, the authors stated that "statistical comparisons were made by standard procedures" (Cerklewski and Forbes, 1976). Table 4-1 indicates that 83% of those studies examined either used no statistics, did not specify the statistical methodology or used Student's t-test.

The other statistical tests employed in these studies are also listed in Table 4-1. Because many studies used more than one procedure, the total number of individual tests is greater than the total number of studies in the survey. Nearly 37% of the studies used a method other than or in addition to the Student t-test. No attempt will be made here to define or characterize each method nor critically assess the appropriateness of these tests for interaction studies except for the use of Finney's (1971) equation (equation 4-8 with p=0) for joint toxic action. Four studies used this additivity model to calculate the predicted LD_{50} values for a number of binary mixtures. Ratios of predicted to observed LD_{50}s were calculated and a determination was made as to the significance of the deviance from additivity. Keplinger and Deichmann (1967) determined the acute toxicity induced by combinations of two and three pesticides and reported that while most of the combinations induced essentially additive effects in mice and rats, there were cases of less than or more than additivity. Pairs of 27 industrial chemicals tested for joint toxic interaction demonstrated that the additive model reasonably predicted the toxicities of a majority of these binary mixtures (Smyth et al., 1969). Departures from additivity were reported by Withey and Hall (1975) who investigated the joint toxic action of perchloroethylene with benzene or toluene and by Freeman and Hayes (1985) who observed the potentiation of acute acetonitrile toxicity by acetone.

A handful of other studies has also attempted to quantify toxic interactions in terms of deviation from an additive response. An undefined additive model was employed by Woolverton and Balster (1981) to investigate the effects of combined ethanol and 1,1,1-trichloroethane exposure.

Wysocka-Paruszewska et al. (1980) used the coefficient of combined action, defined as "the ratio of the calculated LD_{50} on the basis of LD_{50} of a single compound to the experimental LD_{50}" to evaluate the toxicity of thiuram in combination with several other pesticides. Derr et al. (1970) used a response addition approach in which the mean heart or body weights for individual treatment groups (minus control values) were added to calculate the expected combined response to cobalt (cobaltous chloride) and ethanol exposures. The observed and calculated weights were then compared using a Student t-test. The effects of prophylactic protection against cyanide intoxication were evaluated using potency ratios defined as the LD_{50} of KCN with antagonist(s) divided by the LD_{50} of KCN without antagonist(s) (Way and Burrows, 1976). The results of the above studies were varied in that additive, potentiated and antagonistic effects were observed depending on the mixture components and concentrations.

4.6.1. Description of the Mixtures Data Base Sample. The use of statistics in the U.S. EPA mixtures data base has been described in the previous section. A 10% random sample of papers from the U.S. EPA mixtures data base was taken to review the quality of experimental design, use of statistics and ensuing conclusions. The sample was stratified by classification of type of statistics used; there were 32 papers assessed. A detailed critique of these papers is contained in Appendix C. It is important to note that if an investigator used a poor experimental design or inappropriate statistical analyses, the conclusions regarding the interaction are suspect. Unfortunately, it is impossible to determine if the conclusions are correct without access to the raw data for re-analysis.

In summary, there was no use of statistics in 8 studies, the statistics used were not specified in 7, no statistics were given in 2 abstracts, and

no quantitative data were given in 1 paper. Of the remaining papers, the ones that described their statistical methods, the methods used were inappropriate in 9 and there was no baseline control in 4 papers. In one paper, the design and use of statistics were appropriate with the conclusion justified.

4.7. CRITICAL ASSESSMENT EXAMPLE

As a further assessment of the quality of statistical analysis in the mixtures literature, one paper was selected for intensive scrutiny. The study by Eybl et al. (1984) was chosen because of its detailed descriptions of the toxicologic and statistical methods employed.

Eybl et al. (1984) investigated the influence of several chelating agents on the acute toxicity of cadmium (Cd). As will be shown in the following discussion, the experimental design and the statistical methods used were inappropriate for characterizing the interaction for risk assessment purposes, and were in fact inadequate for some of the authors' goals as well. Eybl et al. (1984) examined effects on mice and rats; only the mouse experiments are discussed here. Characteristics common to the mouse test series were as follows:

species:	male mice (SPF, Velaz Prague), 20.22 g body weight
route:	i.v. (single injection)
chemicals:	Cd with any of six chelating agents or combinations
endpoint:	survival rate at 10 days

4.7.1. Experimental Conditions. The first series studied the effect of single chelating agents on survival of mice injected with $CdCl_2$. The conditions were as follows:

Groups:	20 mice per exposure group

Exposure:	toxicant- $CdCl_2 \cdot 2.5H_2O$ single subcutaneous injection 20 mg/kg; inhibiting agent- single intraperitoneal injection at a molar ratio of 25:1 (chelator:$CdCl_2$)
Statistical method:	unspecified, probably Fisher's exact test (Fisher's exact, Chi-square, t-test all mentioned in Methods section)

The conditions for the second, third and fourth series were similar to those of the first series. The second series included three dose levels (molar ratio of 1:1, 2:1, 5:1). The third series used one dose level (molar ratio 5:1) but two treatment sequences (simultaneous vs. 2 hours after the Cd injection). The fourth series used one dose level for single chelator effects and a different dose level for effects of two chelators together. For example, ZnDTPA and DMSA were tested individually at a molar ratio of 2:1, while the combination ZnDTPA+DMSA was tested at a ratio of 1:1:1.

4.7.2. Discussion of Design. This first series seems to have been intended only to screen for the most effective inhibitors (antidotes) of Cd toxicity. Cadmium is always administered at the same dose, and each of the chelators is administered at only one dose level. Consequently, no dose-related interaction can be determined. The authors apparently assume that the data are similar to data on treatment regimens for a disease, where here the disease is Cd toxicity and the treatment is one of the chelators. The "disease-treatment" interpretation, however, requires the assumption that Cd lethality occurs only at 20 mg/kg or more, and that the administered chelator levels are the standard antidote dosages. None of these assumptions has been demonstrated in this paper. Consequently, any conclusions are then specific to the doses used.

No models were presented by the authors as a means of estimating the "expected" response from the Cd-chelator combination. Models of the interaction between a chelator and Cd cannot be applied to the data as presented since results are not given for a control group (no CD, no chelator), nor for exposure to a chelator alone (no Cd). A key unstated assumption is that all the chelators are administered at nontoxic doses.

The statistical test used is not stated, but can be assumed to be the Chi-square or Fisher's exact test. These tests are consistent with the interpretation of the experiment as if it were the treatment of a disease. As further confirmation, use of Fisher's exact test in recalculating the significance levels showed agreement with Eybl's published values (Eybl et al., 1984, Table 1) except for the group 4 to group 6 comparison, which should show $p=0.02$, i.e., it should be footnoted by an asterisk to denote $p<0.05$, not $p<0.01$.

The preceding comments also apply to the other test series. In addition, the doses (molar ratios) used in the second and third series were the same for all chelators, regardless of each one's inhibitory effectiveness. The doses used in the fourth series were selected to provide the same number of moles of mixed chelators as used in each individual test. No model has been located that uses such a dose selection in a mixture study, and the authors do not provide any justification for these doses.

4.7.3. Discussion of Results. The reported results for all four series include the survival fraction (n/20) and significance level (percent) of various differences in survival rate. Several comparisons are made in the first, second and fourth test series with no adjustment for multiple comparisons. The importance of the multiple comparison problem is easily demonstrated with the first series. Note that two comparisons are reported

between chelators, suggesting that all chelator survival rates may have been compared with one another but only a few comparisons were reported. There are 6!/(2! x 4!) = 15 such pairwise comparisons in addition to the six comparisons between each chelator and Cd alone. At a decision significance of 0.05, one of the 21 comparisons can turn out to appear significant through random chance alone. So one of the six significant findings could be circumstantial and not due to actual differences in inhibition. If the decision rule is to require significance of at least 0.05, then the chelators showing survival increase at a significance of 0.01 or lower would probably be significant after the multiple comparison adjustment. The finding that was significant at 0.05 but not 0.01 is suspect. In the fourth series, the multiple comparison issue is not as strong, since only six possible comparisons could be made; the reported significance levels, however, are still inaccurate.

In addition to compensating for multiple comparisons, the analysis should have used survival time (when the animals died) instead of the end survival fraction. In addition to using more data in the statistical analysis, comparing survival curves would have also provided more information for studies on the mechanism and pharmacokinetics of inhibition by the chelators.

Use of a single dose level in the first series is justified for screening purposes. The analysis of the second series should have combined the dose levels, instead of merely reporting pairwise comparisons. For example, if the different series are assumed to be comparable and the groups combined, then the dose-response data appear as in Table 4-2. The dose selection for the fourth series could then have been made according to some interaction model so that the response to the combined chelators could be

TABLE 4-2

Combined Results for CaDTPA and DMSA Inhibition of Cd Toxicity[a]

Chelator	Dose[b]	Survival (%)	Surviving/Total
CaDTPA	1	60.0	12/20
	1	60.6	20/33
	2	80.0	16/20
	5	81.8	27/33
	5	86.7	13/15
	25	85.0	17/20
DMSA	1	3.0	1/33
	2	35.0	7/20
	5	100.0	33/33

[a]Source: Eybl et al., 1984

[b]Dose is molar ratio of chelator:Cd with Cd administered as $CdCl_2 \cdot 2.5H_2O$ at 20 mg/kg

predicted. For example, for a response addition model where independence of action is assumed, the doses used in individual testing would be duplicated in the mixed test (if the molar ratio of chelator:Cd of 2:1 was used for each single chelator test, then the mixed exposure ratio of chelator: chelator:Cd should be 2:2:1). For a dose addition model where similarity of action is assumed, the mixed exposure would use doses scaled according to potency, where the summed scaled dose of the mixture would have been previously tested for one of the single chelators. Instead, since the dose selection was not justified by the authors, and since no predictive model was presented, the authors' conclusion that "the additive effect of these two chelating agents was demonstrated" is false. In general, the conclusions throughout this paper are much weaker than they could have been had adequate design and analysis been implemented.

4.8. SUMMARY

In summary, statistical methods that have been used for assessing interactions among components in chemical mixtures have been examined. This review indicates that proper experimental design is infrequently utilized, and that statistical techniques are rarely chosen appropriate to the experimental data. In particular, current techniques for investigating the presence and extent of interactions in complex mixtures are inadequate, impractical or impossible to apply. At best, practical design and analysis techniques can be applied to characterize interactions in the experimental dose ranges only among constituents of simple mixtures.

5. DISCUSSION AND REASSESSMENT OF THE GUIDELINES

5.1. OVERVIEW

This chapter reviews and reevaluates the current Agency guidelines on mixtures based on the Agency's experience in applying these guidelines as well as considerations of new information that has been obtained and new approaches that have been proposed since the guidelines were developed. Revisions suggested in this chapter along with other comments received by the Agency will be considered for future incorporation into the guidelines.

Based on the mechanistic considerations summarized in Chapter 3, toxic interactions may modify significantly the toxic and carcinogenic potency of environmental contaminants. The types of information available for quantitatively assessing the magnitude of such interactions as reviewed in Chapter 2., however, are not extensive. While appropriate mathematical models and statistical techniques are available to quantify some simple binary interactions, these methods cannot be extended to complex mixtures because the data requirements of such extensions lead to experimental designs that are impractical. In addition, mathematical models for quantifying promotion and cocarcinogenic efficiency that could be used to systematically assess and compare the quantitative significance of these phenomena have not been developed. Those quantitative estimates of compound interactions that can be made suggest that most interactions are within a factor of 10 of those that would be predicted based on the assumption of no interaction. The data on which this generalization is based, however, are limited.

The preferred approach presented in the guidelines for conducting risk assessments on mixtures is to use *in vivo* toxicity data on the mixture itself based on the route of exposure and duration period of concern. This

remains the preferred approach, as long as certain factors such as masking of toxic or carcinogenic effects are considered. Nonetheless, this approach will not be practical in most cases because adequate toxicity data are available on only a few complex mixtures. While the concept of "sufficient similarity" may be able to extend this approach somewhat, this approach will still be restricted to a few well-studied groups of complex mixtures (see Appendix B).

The use of an assumption of dose or response additivity as the basis for risk assessments on mixtures remains a useful, and in many cases the only practical, approach. Some mechanistic considerations suggest that additivity may be a plausible assumption in the low-dose region because thresholds for many types of interactions are expected to exist. In addition, many acute bioassays on binary or simple mixtures suggest that the dose additivity often adequately accounts for mixture toxicity based on gross toxic endpoints. Nonetheless, the credibility of this approach diminishes as the number of components in the mixture increases because for many mixtures the toxicity and perhaps the identity of all components are not known.

Alternatives to any of the above approaches are being developed and explored by the Agency and other groups to more fully utilize the extensive _in vitro_ and short-term _in vivo_ data on many mixtures. Two such alternatives, the "comparative potency approach" and the "toxic equivalency factor," were not discussed in the guidelines.

The "comparative potency approach" attempts to calibrate the _in vitro_ potency of groups of complex mixtures to the limited _in vivo_ potency estimates of these mixtures. Once a relationship between _in vitro_ and _in vivo_ potency has been demonstrated, the results of _in vitro_ assays on other

related complex mixtures can be estimated. As discussed below in Section 5.3., this approach has been applied to the carcinogenic potency of combustion emissions and can be regarded as a more formal and quantitative extension of "sufficient similarity." As with the direct application of sufficient similarity, care must be taken to ensure that the approach is applied only to mixtures that are likely to exert effects by the same mode of action.

The "toxic equivalency factor" method involves estimating the potency of less well studied components in a mixture relative to the potency of better studied components, using data from comparable types of *in vitro* and *in vivo* assays. So far, this method has been used only to estimate the toxicity of mixtures of chlorinated dioxins and dibenzofurans (a group of similar compounds) by using the considerable data on the *in vitro* activity of these compounds. The toxicity of the mixture is then estimated by summing the products of the equivalency factors and concentrations of the components in the mixture. An estimate of the *in vivo* potency of the mixture can be made by multiplying this sum of the products by the *in vivo* potency of the reference compound, i.e., the compound that served as the basis for estimating the toxic equivalency factors (2,3,7,8-TCDD in the case of mixtures of chlorinated dioxins). This approach can thus be regarded as an extension of the assumption of dose additivity and like dose additivity must be restricted to compounds that act by the same mechanism.

Both of the above approaches are likely to prove useful as alternatives or bases for comparison with risk assessments using the hazard index based on dose or response additivity as given in the guidelines. As with any type of analysis based on *in vitro* data, confidence in these methods will vary with the degree to which the *in vitro* analyses have been validated as predictors of *in vivo* responses.

None of the above considerations fundamentally alter the basic approach recommended in the original guidelines. All of these considerations do reinforce the underlying principle of the guidelines: "No single approach can be recommended to risk assessments for multiple chemical exposures. Given the complexity of this issue and the relative paucity of empirical data from which sound generalizations can be constructed, emphasis must be placed on flexibility, judgment, and a clear articulation of the assumptions and limitations in any risk assessment that is developed."

5.2. COMPLEX MIXTURES

For complex mixtures, it is not likely that toxic or carcinogenic interactions will or can be quantified using the mathematical constructs given in Chapter 4. As discussed in Chapter 4 and illustrated in Section 2.4., the types of experimental designs that are required for meaningfully quantifying interactions for single pairs of chemicals are prohibitively complex for the routine assessment of chronic effects. For mixtures containing tens or hundreds of chemicals, the proportions of which can vary over time or among sources of generation, elaborate bioassays for quantifying interactions among components are impractical.

The guidelines currently recommend using data on the mixture or a "sufficiently similar" mixture for the risk assessment. In general terms, the determination of sufficient similarity should consider the chemical composition of the mixture, any variation in the chemical composition, as well as the toxicologic properties of the mixture components and fractions. The criteria for determining "sufficient similarity" are intentionally vague and are likely to vary depending on the nature and quality of the available data, the toxicologic endpoint, and the extent of human exposure. A case study applying the concept of "sufficient similarity" is given in Appendix B. Using this approach, a risk assessment can be conducted if the mixture

on which adequate toxicologic data are available is judged sufficiently similar to the mixture for which a risk assessment is desired. For certain classes of complex mixtures on which human or animal data are available on a relevant route of exposure and are adequate for conducting a quantitative risk assessment (e.g., coke oven emissions), the assessment of "sufficient similarity" should be a useful approach.

For many other classes of complex mixtures, however, such *in vivo* data are not available or if available are not by a route of exposure likely to occur in the environment. As currently written, the guidelines suggest, in the absence of "sufficient similarity," that an additivity assumption be used for similar-acting components after assessing whether data are sufficient for quantifying any component interactions. In practice, this will normally lead to an additivity assumption. If the mixture contains many chemicals, it is also likely that adequate toxicity data will not be available on some of the components. Furthermore, for some highly complex or highly variable mixtures, not all of the chemical components may be known. The Agency recognizes that as the number of components increases and as the number of components lacking adequate toxicity data increases, confidence in the risk assessment diminishes.

The use of a comparative potency method may sometimes be preferable to a simple additivity assumption in cases where the criteria for sufficient similarity are not met. This method, as applied to carcinogens, was presented by Albert et al. (1983) and was further refined by Lewtas (1985). The underlying assumption is that relative potencies among *in vivo* and *in vitro* bioassays are constant:

$$RP_1 = kRP_2 \qquad (5\text{-}1)$$

where RP_1 and RP_2 are the relative potencies of a compound or mixture in bioassays 1 and 2, respectively, and k is a constant. It is also assumed

that a single number is sufficient to characterize the response in each assay and that species show parallel response within an assay. Using these assumptions, the results of *in vivo* mixture bioassays from which quantitative risk assessments can be made are correlated with the quantitative results of *in vitro* bioassays. This correlation can be used as a "calibration curve" to estimate the *in vivo* rate of response of similar compounds or mixtures when only quantitative *in vitro* results are available. Using this approach, Albert et al. (1983) reported that estimates of comparative potency for coke oven emissions, roofing tar and cigarette smoke based on several *in vitro* bioassays (*Salmonella* mutagenicity assay, L5178Y mouse lymphoma cell mutagenicity assay and a sister chromatid exchange assay) were within a factor of <2 of estimates of comparative potency based on epidemiologic data for lung cancer. Using additional data from mouse skin tumor initiation studies, Albert et al. (1983) proposed unit lung cancer risks for diesel and gasoline engine exhaust particulates based on the relative potencies of these particulates in *in vitro* assays. Lewtas (1985) extended this analysis to include emissions from various energy combustion sources.

As discussed by both Albert et al. (1983) and Lewtas (1985), the relative potency approach makes several assumptions concerning mechanisms of action and dose-response relationships among the various types of *in vivo* and *in vitro* bioassays that are used. These assumptions and the corresponding uncertainties must be weighed against the assumption of and uncertainties in dose or response addition. The relative potency approach is attractive because data on the mixture of concern can be generated relatively quickly and inexpensively. In addition, given the increasing amount of data available on the effects of mixtures in *in vitro* tests, as

discussed in Section 2.2., and the dearth of information on the magnitude of toxic interactions *in vivo*, the relative potency method offers one approach to the problem of complex mixtures that is amenable to experimental testing and validation.

The use of the relative potency method or other approaches based on *in vitro* or short-term *in vivo* bioassays seems to be potentially useful for assessing the biologic activity of complex mixtures. Only limited data, however, are available for supporting the quantitative correlation of *in vitro* and *in vivo* relative potencies and the data that are available suggest that the correlation between biological activity in the *in vitro* assay and the *in vivo* assay will not be uniform for all types of mixtures. For instance, *Salmonella* are known to be particularly sensitive to the mutagenic effects of nitropyrene by virtue of the organism's endogenous nitroarene reductase (Mermelstein et al., 1981). A comparative potency judgment of a nitropyrene-containing mixture based solely on *Salmonella* mutation data would likely overestimate eukaryotic mutagenic or tumorigenic activity.

An empirical approach to selecting the most appropriate *in vitro* assay for applying the relative potency approach could be based on the use of a battery of screening tests, including *in vitro* assays and short-term *in vivo* assays (NAS, 1988a). The quality of the correlation in biological activity between the screening tests and the known *in vivo* relative potencies of a related group of complex mixtures could then serve as a guide in determining the most appropriate assays for applying the relative potency method to other related complex mixtures. The scientific validity of applying the relative potency method based solely on empirical correlations is questionable, however, particularly when multiple pair-wise comparisons are made among several *in vivo* and *in vitro* assays. An alternative to multiple

pair-wise comparisons has been proposed by DuMouchel and Harris (1983) using Bayesian statistical methods to combine the results of multiple *in vivo* and *in vitro* assays. Nonetheless, confidence in the use of any *in vitro* or short-term *in vivo* assay for estimating environmental risk will depend on the extent to which the assay reflects the mechanism of action and pharmacokinetics of the mixture. For many *in vitro* assays, which provide only an exogenous activating system, this confidence may be limited.

Furthermore, in many instances, the dose-response curves within *in vivo* or *in vitro* assays for even pure chemicals are not linear over a wide range of concentrations or doses. Consequently, a single meaningful "potency" term will not be appropriate for comparing arrays of nonlinear curves. If the "potency" is expressed as an estimate of single slope parameters taken from the mid-range or linear portion of the dose-concentration curve of the *in vitro* bioassay and such values are correlated with linearized potency terms from *in vivo* bioassays with relatively few dose groups and small numbers of animals per group, the errors associated with the estimated potencies are likely to be high and the significance of any correlation questionable.

Notwithstanding these limitations and concerns, the use of the comparative potency method or some analogous approach based on *in vitro* or short-term *in vivo* tests may be the only practical method for assessing risks posed by complex mixtures on which adequate long-term *in vivo* studies are not available. The extent to which the use of such an approach can be considered scientifically valid or simply the application of a risk management decision scheme is likely to vary depending on the quality of the correlations in biological activity and the degree to which a clear association can be made between mechanisms of action in the screening assays and in

the *in vivo* effect induced by the mixture. Depending on the number of compounds in the mixture of concern and the adequacy of the toxicologic data on these compounds, it may be most reasonable to use both the comparative potency method as well as the assumption of dose or response additivity to gauge the variability between the two methods and better express the uncertainty in the risk assessment.

This approach has generally been applied only to carcinogenic effects. An application to noncancer health effects could be reasonably made if the mechanisms of action were similar between the effect of concern and the *in vitro* or short-term *in vivo* bioassays proposed and if data were adequate for assessing the constancy and the correlation in potencies between the short-term and long-term assays.

5.3. MIXTURES OF CHEMICAL CLASSES

As discussed in Section 2.2., mixtures of chemical classes differ from complex mixtures in that the compounds in the former category are structurally and toxicologically related. Some types of mixtures of chemical classes are produced and used as a mixture following a reasonably consistent and well-defined procedure. Examples of such mixtures include the various commercial polychlorinated and polybrominated biphenyls, toxaphene and chlorinated naphthalene. Other types of such mixtures are chemically and toxicologically related compounds that are usually found together in the environment but can vary substantially in the proportions of the components depending on the source of the mixture. Examples of these latter mixtures include polychlorinated and polybrominated dioxins and dibenzofurans. This distinction between these two mixture types is intended to reflect the different types of data that are available or might reasonably be obtained on mixtures of chemical classes.

Because some mixtures are reasonably consistent and limited in the diversity of their composition, data are available on their different commercial formulations (e.g., Aroclor 1264). When data are not available on a specific formulation, the formulation lacking data may often be sufficiently similar to a formulation for which data are available so that a risk assessment can be conducted by analogy. For such mixtures, it thus seems reasonable to continue to conduct risk assessments using toxicity data on the mixture as the preferred approach. Nonetheless, data may sometimes suggest that differential rates of environmental decay or environmental partitioning of the mixture components may lead to human exposures to a mixture that is not representative of the mixture on which the risk assessment was originally based. In such cases, quantitative structure activity relationships or approaches based on the relative potency method discussed above may have merit. Such modifications to the current approach have not been conducted as yet by the Agency and examples of such approaches have not been encountered in the literature. If such approaches are used, their validity will be dependent, as with the relative potency approach, on the degree to which the approach can be validated with in vivo data.

Other mixtures, such as the chlorinated dioxins and dibenzofurans, require a different approach since "typical" formulations or compositions do not exist and thus the multiple chronic bioassays may be not be feasible. The Agency has proposed an interim procedure for estimating risks associated with exposure to chlorinated dioxins and dibenzofurans (U.S. EPA, 1987c). A similar approach has been used by the New York State Department of Health (Eadon et al., 1986). As with the relative potency approach, these methods rely on in vitro or acute in vivo data. Rather than using such data to assess the toxicity of the mixture of concern, however, these approaches

estimate "toxic equivalency factors" for the various congeners in the mixture based on acute or in vitro data and validate the relationship with the available data on chronic or subchronic toxicity. The toxic equivalency factors can then be used to assess the hazard posed by exposure to any combination of the congeners in any ratio. To do this, the concentration of each component in the mixture is multiplied by the toxic equivalency factor of that component. This product expresses the concentration of the component as an equivalent concentration of the reference compound. The equivalent concentrations for all components are then added. This total represents an estimate of exposure to the mixture in terms of the reference compound. This transformed exposure estimate is then multiplied by the potency of the reference compound (2,3,7,8-TCDD in the case of the chlorinated dioxins) to obtain an overall estimate of risk. Depending on the quality of the monitoring data and exposure assessment, U.S. EPA (1987c) also provides recommendations for modifying the risk assessment. As reviewed by U.S. EPA (1987c), several other countries and organizations have adopted similar approaches for the chlorinated dioxins.

The relative potency approach and the toxic equivalency approach are similar in that both use types of data to assess and quantify the toxicity of mixtures that are not often used to quantify the risk from exposure to single chemicals (i.e., acute data, data from atypical routes of environmental exposure and in vitro data). They differ, however, in that the toxic equivalency approach rests explicitly on the assumption of dose or response additivity; this method should be applied only to compounds that have the same mode of action or act independently, and does not account for any potential interactions. If significant interactions do occur in the

mixture, as appears to be the case with the promotion efficiency of polybrominated biphenyls (Sleight, 1985), the toxic equivalency approach could result in risk assessments that are misleading.

The relative potency approach, while not explicitly based on simple similar action, assumes a linear nonthreshold response as it is applied to carcinogens by Albert et al. (1983). In that the relative potency method, however, is conducted on mixtures and validated using in vivo data on mixtures, the possibility to account for interactions is not excluded. A combination of the relative potency and toxic equivalency approaches could improve confidence in risk assessments of similar mixtures and mixtures of chemical classes.

In applying either the relative potency or the toxic equivalency factor methods, care must be taken to ensure that the compounds are not only chemically but also biologically similar. Taking an example from Mehlman and Witz (1986), a mixture of ketones containing methyl-n-butyl ketone and methyl isobutyl ketone would be similar only superficially because methyl-n-butyl ketone, unlike methyl isobutyl ketone, is a potent peripheral neuropathic agent. The failure to account for the neurotoxic potency of methyl-n-butyl ketone, which is toxicologically more similar to n-hexane and 2,5-hexadione than to other ketones, could lead to an erroneous risk assessment. While this type of potential error can occur in dealing with single chemicals with an incomplete data base (e.g., lack of a teratogenicity study), the potential for this type of error is higher when dealing with mixtures and using data that are normally considered inadequate for conducting risk assessments on single compounds.

5.4. SIMPLE MIXTURES, COMPONENTS AND TOXIC INTERACTIONS

In the guidelines for mixtures, the Agency has proposed using additivity assumptions when data are not available on the mixture of concern or a

reasonably similar mixture, and when the components are mechanistically similar or independent. For toxic agents with thresholds, a Hazard Index (HI) is recommended based on the assumption of dose additivity, and can be expressed as follows:

$$HI = E_1/AL_1 + E_2/AL_2 + \ldots + E_n/AL_n \qquad (5\text{-}2)$$

where E is the level of exposure and AL is the acceptable level of exposure. The reference dose (RfD), an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime, is recommended for use as the "acceptable level" (AL) in order to standardize Agency risk assessments. Since HI is dimensionless, use of the RfD means that exposure (E) must then be presented in similar units as daily intake (mg/kg/day). For carcinogens, the recommended equation is based on a simple addition of risks. At low risk levels, this equation simplifies to

$$P = D_1B_1 + D_2B_2 + \ldots + D_nB_n \qquad (5\text{-}3)$$

where P is the expected response, D is the dose (level of exposure) and B is an estimate of response rate (usually a plausible upper bound called a slope factor). In the low-dose region where responses are linear, equation 5-3 is considered to be a reasonable approximation. At higher levels of risk, nonlinearity and competing risks would need to be considered. In addition, the guidelines also suggest some simple interactive models by which nonadditive joint action could be considered, while recognizing that adequate data for using such models will usually not be available.

Since the publication of the guidelines, the literature on joint action has not suggested any fundamental revisions to the above approach. Berenbaum (1985a) has suggested a general approach estimate of expected

responses under the assumption of additivity. Seiler and Scott (1987) illustrated a method for partitioning attributable risks under either the assumption of additivity or using data adequate for quantifying interactions. The available data base on the magnitude of toxic interactions for environmental contaminants has not, however, changed substantially. In most cases, an estimate of risk for exposure to a chemical mixture will be based on an additivity assumption, except in those cases where chronic mixture data or an appropriate surrogate approach (e.g., relative potency) are available.

The additivity assumptions presented in equations 5-2 and 5-3 do, nonetheless, have serious shortcomings. As applied to toxicants, equation 5-2 implies that as the acceptable level is approached or exceeded, the level of concern increases linearly (e.g., an HI of 50 is of twice as much concern as an HI of 25) and in the same manner for all mixtures. As the mixtures guidelines note, these implications are incorrect. RfDs (the values recommended for use as acceptable levels) do not have equal accuracy or precision, and are not based on the same severity of toxic effect. Moreover, slopes of dose-response curves in excess of the RfD in theory are expected to differ widely. The determinations of accuracy, precision or slope are exceedingly difficult because of the general lack of toxicity data. Severity of endpoint, however, is often known. For example, with fluoride and selenium it is known that relatively narrow excursions above or below the RfD can cause severe adverse effects through toxicity or deficiency, respectively. Among other compounds, the margins of safety or error are thought to vary because of differences in the quality of the available data or the relationships of dose and time of exposure to the incidence, severity or intensity of effects. Some of these sources of variability and uncertainty have been discussed in the literature (Crump, 1984; Dourson and Stara, 1983; Lu, 1985; Rulis, 1987), but approaches to

quantifying these differences among chemicals have not been adopted for single compounds, and this inhibits modification and improvement of the current approach for the assessment of mixtures of systemic toxicants.

For carcinogens, equation 5-3 may be overly conservative because upper bounds rather than estimates of expected risk are added. This limitation is recognized but a practical alternative has not been proposed. As discussed in the Agency's guidelines for carcinogens, upper bounds on risk are used because of the substantial uncertainties involved in high- to low-dose and species-to-species extrapolation. Conversely, as discussed by Berenbaum (1985b), synergistic interactions between carcinogens may result in dose-response curves that are steeper in the low-response region than in the experimentally observable region. In such cases, the assumptions of linearity and additivity could underestimate risk. This can also be the case in heterogeneous responding populations (Margosches et al., 1981). Mechanisms for low-dose synergism have not been proposed; in fact, Thorslund and Charnley (1987) show that under the multistage theory, experimentally determined synergism will not significantly differ from the low-dose risk estimate based on additivity.

5.5. MIXTURES OF CARCINOGENS WITH OTHER COMPOUNDS

The enhancement (by promotion or cocarcinogenicity), inhibition or masking of the carcinogenic activity of known or unidentified carcinogens in complex mixtures is only briefly discussed in the Agency's guidelines on mixtures. While potentially of great practical importance (Reif, 1984), few specific proposals have been made to assess and quantify such interactions.

Even with all the work that has been done on tumor promoters and cocarcinogens, much of which is summarized by Lucier and Hook (1983), systematic

and predictive relationships for expressing and measuring enhancement have not yet emerged. Given the complexities of promotion/cocarcinogenicity, it is not surprising that no clear approach for incorporating these concepts into a risk assessment methodology has been recommended. While some approaches to low-dose extrapolation have been recommended which consider the effect of promoters on the initiator dose-response relationship (Burns et al., 1983), no dose-response models that consider variations in both doses of the initiator and doses of the promoter have been proposed or applied to complex mixtures. As discussed by Stara et al. (1983), several questions must be answered before such applications are likely to be made:

> How specific and consistent are initiator-promoter interactions? Does the promoting efficiency of a compound vary with initiating agents and, if so, does this variation follow a consistent or predictable pattern?
>
> How does exposure to multiple promoting agents affect the promoting efficiency of the individual promoters? If additivity is a reasonable assumption, which type of additivity might be expected based on what we know about the mechanism of promotion?
>
> How does promoting efficiency vary with the duration of exposure to the initiator and the promoter?
>
> Is there any validity in using promotion data from one route of administration to predict promoting activity from another route of exposure?

These questions remain largely unanswered. Until answers or reasonable assumptions are proposed, progress in directly applying promotion/cocarcinogenicity data to quantitatively modifying risk assessments for mixtures is likely to be minimal.

A similar situation exists with compounds that cause an apparent inhibition of or protection from chemically-induced carcinogenicity. As reviewed by NRC (1980), very few examples of this type of interaction have been noted and the nature of the interaction can vary with the time course of exposure.

More recently, in reviewing the literature on tumor promotion of the liver, Hermann (1985) cites a few additional studies showing a decrease of preneoplastic liver foci after prolonged treatment with some anti-oxidants or hypolipidemic compounds and suggests that such "anti-promoters" may have potential in the control of cancer. While such a prospect is encouraging, the data currently available are not sufficient for quantifying the dose and time relationships for tumor inhibition. Until such data become available, the presence of tumor inhibitors in mixtures are not likely to be used in quantitatively modifying the risk assessment unless they are incorporated in a comparative potency assessment.

The problem of masking of the carcinogenic activity of some components in a mixture that is due to simple dilution or to the toxic but noncarcinogenic activity of other components in the mixture is less difficult to address than either enhancement or inhibition of carcinogenicity. One component of this problem simply is to account for competing risks. As in the example cited in Section 2.5. from Raabe (1987), this problem is not unique to mixtures. The statistical methods for accounting for competing risks in animal bioassays are available in the literature (Altschuler, 1970; Hoel, 1972; Peto et al., 1972; Peto, 1974) and are incorporated into some commercially available statistical programs for the analysis of cancer bioassay data (e.g., MULTI-WEIB by Howe and Crump, n.d.). In other instances, an adequate chronic study showing no carcinogenic activity may be available on a mixture that contains known carcinogens. While the guidelines state that data on the mixture of concern are preferred to additivity assumptions based on the known activity of the components in the mixture, the analysis of such a "negative" bioassay must consider whether a carcinogenic response would have been expected given the doses and numbers of

experimental animals used. As with masking due to toxicity, masking due to dilution is not unique to mixtures but is essentially identical to evaluating the significance of negative and positive results from different bioassays of a single compound.

6. RESEARCH NEEDS

For complex mixtures, similar mixtures and mixtures of chemical classes, the kinds of research needs vary depending on the specific approach to be taken in developing the risk assessment. For instance, U.S. EPA (1987c) proposed the following research needs for better validating the toxic equivalency factor approach for chlorinated dioxins and dibenzofurans:

1. Validation and completion of *in vitro* test data.
2. Investigation of the relationships between short-term *in vivo* and *in vitro* tests and the chronic toxic endpoints of concern (i.e., carcinogenicity, reproductive toxicity, immunotoxicity and other significant human health effects).
3. Additional data on pharmacodynamics and metabolite toxicity.
4. Development of additional short-term assays which can test the mechanistic hypotheses underlying the toxic equivalency factor approach.

Since this approach may also prove useful for other classes of compounds, such as the brominated dioxins and dibenzofurans, comparable studies on these classes of compounds might also be added to the above list.

Research needs for the comparative potency method are somewhat similar. Currently, the relationship is validated by comparing the *in vitro* and *in vivo* relative potencies of relatively few mixture classes. Confidence in this method could be improved if the basis for the comparison was broadened to include not only relative potency estimates from human studies but also potency estimates from animal bioassays. In addition, a more extensive comparison including not only data on mixtures but also data on individual compounds would help to strengthen this approach.

Both the toxic equivalency factor and comparative potency methods are generally applied only to carcinogens. While the *in vitro* tests on which

these methods are currently based are probably only appropriate for carcinogens, other short-term assays have been developed for other endpoints (e.g., teratogenicity and cytotoxicity) that may be applicable to the assessment of the noncarcinogenic toxicity of mixtures. Given the diversity of mixtures in the environment, the validation of a battery of short-term assays to assess the systemic effects of mixtures could serve as a valuable adjunct to the additivity assumption.

In implementing this research, the validation of screening tests must be recognized as a complex process. As discussed with respect to several kinds of assays (Brown et al., 1979; Purchase et al., 1976; Rinkus and Legator, 1979, 1980; Rosenkranz and Poirier, 1979; Sugimura et al., 1976) validations require not only careful criteria for assessing false positive and false negatives but also a consideration of the class of compounds used to validate the assay and the limitations that this may impose on the usefulness of the assay for other classes of chemicals. In addition, the proposal to use any screening test is greatly supported by the demonstration that the mechanisms of action are similar for the toxic effect of concern and the response observed in the screening test. Depending on use and consistency of the screening test, greater attention may need to be given to the statistical analyses of the assay results (Gart et al., 1979; Frome and DuFrain, 1986) so that the errors and uncertainty in any analysis can be more explicitly identified.

As noted in Chapter 5, the use of the additivity assumption is somewhat restricted by the approach currently used for risk assessment of single systemic toxicants. While an improvement of this situation appears to be more a matter of analysis than the generation of additional data, it is an area that must be addressed if an improvement in the application of the assumption of additivity is to be made.

As discussed in Section 2.6. and Chapter 4, several noninteractive models can be applied to the diverse kinds of quantitative interaction data that are available. In addition, appropriate statistical methods have not been applied to much of the data that are available, and the limitations of some of the available information preclude the application of any quantitative model. Consequently, no generalizations can be made on the quantitative significance of interactions at normal environmental levels. This problem could be at least partially addressed by a detailed reanalysis of the available data by applying a variety of noninteractive models to derive quantitative interactive coefficents.

In conducting risk assessments for single compounds, both carcinogens and systemic toxicants, the Agency uses conservative but plausible assumptions concerning extrapolations from high to low doses and species to species, and concerning modeling of time-to-effects data. Concern has been expressed both within the Agency and by other elements of the scientific community that the use of dose or response additivity combining such conservative risk assessments for individual chemicals could lead to implausibly conservative risk estimates for complex mixtures. This limitation in the use of an additivity assumption is one of the reasons that the Agency prefers using data on the mixture of concern or a sufficiently similar mixture and has used the relative potency method or toxic equivalency factor approach for complex mixtures. Nonetheless, additivity assumptions will be used for many risk assessments on mixtures, and the need to develop alternative risk assessment procedures or testing strategies is recognized. The Agency is currently reviewing the recent recommendations of NAS (1988a) along with other approaches.

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APPENDIX A

AGENCY DATA BASE ON MIXTURE TOXICITY

OVERVIEW

The toxic interaction data base contains information obtained from literature searches of all published studies on interactions between toxic chemicals (U.S. EPA, 1988). The goal is to be complete, not merely representative, so that analysis of the data, e.g., for trends across chemical classes, can be performed if desired. This version does not contain extensive quantitative data. This constraint is consistent with the Agency Guidelines for the Health Risk Assessment of Chemical Mixtures (U.S. EPA, 1986), which do not recommend any quantitative method for including interaction data into a risk assessment. As a result, the current version of the data base is most useful for a qualitative evaluation of the potential types of toxic interaction between two environmental chemicals.

The data base package contains a User's Guide, diskettes (IBM PC compatible), and a table for interpreting the CASSI codes (CAS, 1980) for the reference citations. The data base is in dBASE III Plus format. The access programs are compiled dBASE programs, and can be run without the need for dBASE III Plus. The data base is available from the Risk Assessment Contacts in each of the U.S. EPA's Regional Offices.

DATA BASE STRUCTURE

The data base includes 13 data fields, which are described in detail in the next section. The structure is as follows:

Field Name	Type	Width	Description
CAS-ONE	Character	12	CAS No. of first chemical
CMPD-ONE	Character	30	First chemical name
CAS-TWO	Character	12	CAS No. of second chemical
CMPD-TWO	Character	30	Second chemical name
RTE-EXP	Character	7	Exposure route
SPECIES	Character	7	Animal species
SEQUENCE	Character	8	Treatment sequence
DUR-EXP	Character	7	Exposure duration
SITE	Character	7	Site of adverse effects
EFFECTS	Character	10	Type of adverse effects
INTERACT	Character	7	Type of interaction
AUTHOR	Character	30	First two authors (or et al.)
REFERENCE	Character	26	Reference code, volume:page

An example of input format and corresponding on-screen computer display is illustrated in Figure 1.

DESCRIPTION OF DESIRED FIELDS

Compounds

Each compound is listed as either Compound I or Compound II.

CAS Numbers

CAS numbers corresponding to the above mentioned compounds are included.

Routes of Exposure (codes provided - Table 1)

The routes of administration are specified for each compound and are listed in order, i.e., Compound I first and Compound II second. For example, in the study by Short et al. (1977), vinylidene chloride was given via inhalation while disulfiram was administered orally. In Figure 1, this is illustrated as follows:

IHL; ORL

a) Input Data Sheet

Compound I: Vinylidene chloride CAS No.: 75-35-4

Compound II: Disulfiram CAS No.: 97-77-8

Route of Exposure: IHL; ORL

Species: MUS

Treatment Regimen: II; I and SIM

Duration: ACU

Site: WBY

Effects: MOR(I)

Qualitative Assessment: INH

Reference: Short, R.D., Winston, J.M., Minor, J.L., Hong, C., Seifter, J., and Lee, C. 1977. Toxicity of vinylidene chloride in mice and rats and its alteration by various treatments. J. Toxicol. Environ. Health. 3: 913-921.

b) Corresponding On-Screen Display

75-35-4 VINYLIDENE CHLORIDE
97-77-8 DISULFIRAM

Route: IHL;ORL Species: MUS Sequence: II;I&SIM Duration: ACU
Site: WBY Effects: MOR(I) Interaction: INH

Author: SHORT,RD ETAL Ref: JTEHD6 (1977) 3:913-21

FIGURE 1

Example of Interaction Data Showing Original Coded Data and On-Screen Representation

TABLE 1

Route of Exposure

GAV	- gavage	IRN	- intrarenal
IAT	- intraarterial	ISC	- intrascapular
IAL	- intraaural	ISP	- intraspinal
IBR	- intrabronchial	ITR	- intratracheal
ICE	- intracerebral	IVG	- intravaginal
ICV	- intracervical	IVN	- intravenous
IDR	- intradermal	OCU	- ocular
IDU	- intraduodenal	ORL	- oral (dietary)
IHL	- inhalation	PAR	- parenteral
IMP	- implant	REC	- rectal
IMS	- intramuscular	SCU	- subcutaneous
IPC	- intraplacental	SKN	- skin
IPL	- intrapleural	UNR	- unreported
IPR	- intraperitoneal		

When the same exposure route is used for both compounds, the route is listed only once. For example, if lead and zinc were both administered orally, the input would read

ORL

Species (codes provided - Table 2)

The species utilized in the study of interest

Treatment Sequence

This field specifies whether the compounds of interest were administered simultaneously or sequentially. If administration was sequential, the order of administration is specified by the number of the compound. In Figure 1, "treatment regimen" indicates that disulfiram was administered before (II; I) and simultaneously with (SIM) vinylidene chloride. If the two compounds had been administered concurrently, the format would read

SIM

Duration of Study

The duration of the study of interest is classified as either acute, subchronic, chronic or lifetime where

acute = $\leq$14 days (ACU)

subchronic = >14 days but not $\leq$90 days (SCH)

chronic = >90 days (CHR)

lifetime = lifetime (LIF)

Sites (codes provided in Table 3)

The site or sites affected by the compound of interest are entered in this field. In Figure 1, the observed endpoint was decreased survival, which is considered a whole body effect. Thus, the site of the effect is coded as WBY. Duration is defined as the period between the beginning of

TABLE 2

Species

CAT	-	cat
CTL	-	cattle
CHD	-	child
DOG	-	adult dog
DOM	-	domestic animals (goat, sheep, horse)
GRB	-	gerbil
GPG	-	guinea pig
HAM	-	hamster
HMN	-	human
INF	-	infant
MKY	-	monkey
MUS	-	mouse
PIG	-	pig
RBT	-	rabbit
RAT	-	rat
SQL	-	squirrel

TABLE 3

Site/Organ Affected

ADR	adrenals	LVR	liver
BLD	blood	LYM	lymphocyte
BMR	bone marrow	MMB	mucous membrane
BRN	brain	MSK	musculoskeletal
BRS	breast	MTH	mouth
CAR	carcass	NSL	nasal passageways
CER	cervix	OVR	ovary
CNS	central nervous system	PAN	pancreas
COL	colon	PLC	placenta
CVS	cardiovascular system	PNS	peripheral nervous system
EAR	ears	PUL	pulmonary system
EMB	embryo	RBC	red blood cells
END	endocrine	SEN	gen. sensory
EYE	eyes/ocular	SKN	skin
FAT	fatty tissue	SOI	site of injection
FET	fetus	SPL	spleen
GEN	genitals (external)	TES	testes
GIT	gastrointestinal tract	THM	thymus
HED	head	THR	thyroid
HRT	heart	UNS	unspecified
KDN	kidney	UTS	uterus
LIM	limbs	WBY	whole body
LNG	lung		

treatment and the time when the endpoint assay is conducted. In teratology studies, exposure during gestation is considered chronic to the life of the fetus.

Effects (codes provided in Table 4)

The effects observed at the above-mentioned site or sites. In cases where only one compound produces an effect (potentiation, no apparent interaction, inhibition), the compound number is placed in parentheses after the code for effect.

For example, in Figure 1, the effect of interest is a vinylidene chloride-induced increase in mortality (MOR). Thus the "effects" field reads

MOR(I)

In cases where both compounds cause an effect at a given site (antagonism, additivity, synergism) or opposite effects at a given site (masking), the interacting compounds are not listed in parentheses after the effect.

Type of Interaction

In an attempt to characterize toxicant interactions, a scheme of classification (see Figure 2 for an outline) has been devised to distinguish between the various types of interactions encountered in the existing literature. The scheme is as follows:

Both Compounds I and II Produce a Given Effect at a Given Site.

1) Additive - The magnitude of the effect observed in the presence of both compounds is not quantitatively greater or less than the sum of effects produced by each compound alone. For example, both aldrin and aramite cause increased mortality when administered individually to mice. When administered together, the observed mortality is equal to the sum of mortalities observed for each compound administered individually (simple response addition).

TABLE 4

Nature of Effect

ABS	- absorption altered	MOR	- mortality
ALR	- allergic responses (i.e., hypersensitivity)	MUT	- mutagenic
COR	- corrosive effects (burns, desquamation)	NBH	- neurobehavioral effects
DDP	- drug dependence	NEO	- neoplastic
DEG	- degenerative changes	NPY	- neuropathy
DEP	- depression of function	OCC	- ocular effects
DIS	- distribution altered	PIG	- pigmentation changes
ELI	- elimination altered	PRO	- proliferative changes
ENZ	- enzyme activity altered	REP	- reproductive effects
EXC	- excretion altered	RET	- retention altered
FUN	- functional impairment	STI	- stimulation of function
HEM	- hematologic changes	SUR	- survival/viability altered
HMR	- hemorrhage	TEM	- temperature changes
IRR	- irritation	TER	- teratogenic
MET	- metabolism altered	UNS	- unspecified effects
		WGT	- weight altered

A. Both compounds produce a given effect at a given site or sites

   1. Additive (ADD)
   2. Antagonism (ANT)
   3. Synergism (SYN)

B. Only one compound produces a given effect at a given site or sites

   1. Inhibition (INH)
   2. No Apparent Influence (NAI)
   3. Potentiation (POT)

C. Neither compound alone produces a given effect but when placed together, an effect is seen - Chemical Synergism (CSY)

D. Compounds I and II produce opposite effects at the same site or sites - Masking (MSK)

E. Unable to assess (UTA)

FIGURE 2

Types of Interaction with Codes

2) Antagonism - The magnitude of the effect in the presence of both compounds I and II is less than would be expected in the case of additivity. For example, both 2,4-D butyl and 2,4,5-T butyl produce teratogenic effects and fetal mortality when administered alone; however, the effects seen when both compounds are administered together are less severe than those seen when either 2,4-D butyl or 2,4,5-T butyl is administered alone, and hence, less than expected under the additivity assumption.

3) Synergism - The effect seen in the presence of both compounds is quantitatively greater than would be expected in the case of additivity. For example both PCB and vinylidine fluoride cause an alteration in enzyme activity in the liver when administered individually. When administered together, the effect is quantitatively greater than would be expected in the case of additivity.

Only One Compound Produces a Given Effect at a Given Site.

The following three classifications are special cases of the three discussed previously: addition, antagonism and synergism.

1) No Apparent Influence - A noneffective compound, II, does not modify ostensibly the effect produced by compound I. For example, acrylamide-promoted neuropathy is unaffected by the co-administration of cortisol. Cortisol alone has no effect upon the peripheral nervous system. Thus, cortisol has no apparent influence on the acrylamide-promoted neuropathy.

2) Inhibition - The noneffective compound, II, quantitatively inhibits the effect produced by Compound I. An example of inhibition is presented in Figure 1. Vinylidene chloride (compound I) caused an increase in mortality, which was inhibited by co-administration of disulfiram (compound II). When administered alone, disulfiram had no effect on the survival rate; thus, the interaction was classified as inhibition rather than "antagonism" or "masking."

3) Potentiation - The noneffective compound, II, enhances the magnitude of the effect produced by compound I. An example of potentiation is vinylidene chloride-promoted degenerative changes in the liver, which are enhanced quantitatively by the co-administration of acetone. Under the conditions of the experiment, acetone alone has no effect upon the liver.

Masking

The assessment of "masking" is reserved for the instance when compounds I and II produce opposite effects at the same site or sites and either diminish or override the effects of each other. For example, zinc alone has been shown to cause an increase in S-aminolevulinic acid dehydratase activity (ALA-D) in red blood cells, while ethanol alone causes a decrease in ALA-D in red blood cells. Co-administration results in a rise in ALA-D quantitatively similar to that observed when zinc was administered by itself. Thus, on the input sheet, "Effects" would read "ENZ" and qualitative assessment would read "MSK."

Unable to Assess

This is used for studies that are poorly designed or insufficiently detailed to discern the nature of the interaction.

Reference

The input data sheets contain the complete reference (see Figure 1 for an example). The data base includes only the first two authors (second author is "et al." if more than two authors), year, reference code and volume:page numbers.

GENERAL COMMENTS

In most cases, identical data generated by the same laboratory but reported in more than one reference were not repeated in the data base. In addition, results reported in the text without accompanying data were not used because an adequate evaluation of the interaction could not be made. This data base is only concerned with effects resulting from excess exposures, e.g., studies examining the consequences of feeding diets deficient in an essential nutrient were not included.

In general, because of a widespread lack of adequate statistical methodology in the studies reviewed, assessing the qualitative relationships between compounds was often difficult. In many cases, it was left to the judgment of the reviewer whether an interaction existed at all and, if so, how to classify it according to the scheme presented above. It should be emphasized that this data base should be used only as a tool to direct the user to the literature currently available regarding toxicant interactions.

APPENDIX B

DIESEL EXHAUST EMISSIONS AND "SUFFICIENT SIMILARITY"

DIESEL EXHAUST EMISSIONS AND "SUFFICIENT SIMILARITY"

An important concept in the Guidelines for the Health Risk Assessment of Chemical Mixtures (U.S. EPA, 1986) is the use of data on similar mixtures for a risk assessment on the mixture of concern. This procedure is predicated on the determination of "sufficient similarity" between the mixtures. In brief, if health effects data on a similar mixture are available, it must be determined if the mixture on which there are data is or is not sufficiently similar to the mixture of concern to allow a risk assessment. This determination should include consideration of the component proportions of the mixtures as well as any toxicologic or pharmacokinetic data on the components or the mixtures that would assist in assessing the significance of any chemical differences between the similar mixture and the mixture of concern. The determination of "sufficient similarity" should be made on a case-by-case basis in light of the uncertainties associated with using data on a dissimilar mixture and with using other approaches such as additivity. (For further information concerning the applicability of the sufficient similarity approach, refer to the guidelines.)

Diesel engine exhaust represents a family of complex mixtures that are generated with varying compositions depending on different temporal, emission source, or operating condition characteristics. Because diesel engine emissions were expected to make a significant contribution to urban pollution, the U.S. EPA instituted a major research program aimed at quantifying the potential health and environmental impacts of diesel-powered light-duty vehicles (U.S. EPA, 1979). The purpose of this exercise is to use these data to determine whether diesel emissions from different

sources are sufficiently similar to warrant their use for the purposes of predicting the health effects of unknown diesel emissions as outlined in the guidelines.

The U.S. EPA research program was designed to determine the relative mutagenic and carcinogenic potency of extractable organics from diesel particulate emissions compared with particle-bound organics from other environmental emissions (gasoline engines, cigarette smoke condensate, and coke oven and roofing tar emissions) (Lewtas et al., 1981). The mobile source samples selected for this study included a heavy-duty Caterpillar 3304 diesel engine, three light-duty diesel passenger car engines (Datsun Nissan 220C, Oldsmobile 350, and Volkswagen turbocharged Rabbit), and a gasoline catalyst Mustang II. All diesel engines were operated on the same lot of No. 2 diesel fuel. In addition, all vehicles (except for the Caterpillar) were operated on a chassis dynamometer under identical conditions using the highway fuel economy test cycle (HWFET). Particle samples from all engines were collected with a dilution tunnel in which the hot exhaust was diluted, cooled, and filtered through Pallflex T filters. All samples were extracted by a Soxhlet apparatus with dichloromethane, which was removed by evaporation under dry nitrogen.

The test matrix consisted of the following bioassays: reverse mutation in Salmonella typhimurium; sister chromatid exchange (SCE) in Chinese hamster ovary (CHO) cells; gene mutation in L5178Y mouse lymphoma cells and BALB/c 3T3 (3T3); viral enhancement of transformation in Syrian hamster embryo (SHE) cells; oncogenic transformation in 3T3 cells; and skin tumor initiation in SENCAR mice. Where possible, these bioassays were conducted such that a positive dose-response relationship was observed over at least three doses above spontaneous levels. Comparative potency rankings of the

samples were determined based on the initial linear slope of the response curve. Where dose-response data were not obtained, the lowest effective dose (LOEL) tested was determined.

A wide range of activity was observed in S. typhimurium strains TA98 and TA100 (Table B-1). The majority of the activity associated with the diesel samples was direct acting while the addition of a mammalian activation system increased the activity of the gasoline engine sample (Claxton, 1981).

All of the emission samples gave positive mutagenic responses both in the presence and absence of metabolic activation using the criteria of the L5178Y mouse lymphoma thymidine kinase (TK) locus forward mutation assay. The diesel engine emissions were more cytotoxic in the absence of metabolic activation while cytotoxicity increased in the presence of activation with the Mustang emissions. Among the diesel engines, the Nissan emission sample was the most cytotoxic while the Caterpillar sample was the least cytotoxic with a potency below that of the gasoline engine (Mitchell et al., 1981).

Curren et al. (1981) assayed the Caterpillar, Nissan, and Oldsmobile diesel samples, and the Mustang gasoline sample in the BALB/c 3T3 mutagenesis assay. Although several individual doses of the diesel extracts induced a significant increase in ouabain-resistant mutants, none of the samples yielded a dose-dependent increase in mutation frequency. Based on a determination of mutation frequency for the dose ranges tested, both the Nissan and Mustang samples were significantly mutagenic both with and without metabolic activation while the Caterpillar and Oldsmobile samples were not significantly different from controls.

Definitive conclusions concerning the DNA-damaging capabilities of diesel emissions as measured by the SCE test are difficult to reach given

TABLE B-1

Specific Activities at 100 µg of Organic Material* in Salmonella typhimurium Strains TA98 and TA100

Sample	TA98		TA100	
	+S9	-S9	+S9	-S9
		Diesel		
Caterpillar	59.3	65.9	115.2	167.8
Nissan	1367.1	1225.2	881.7	1270.1
Oldsmobile	318.7	614.8	169.9	247.5
VW	297.5	399.2	426.0	641.6
		Gasoline		
Mustang	341.9	137.8	228.0	196.5

*Source: Claxton, 1981

that the results are based on single experiments. However, it is significant that an observed increase in SCE frequencies in CHO cells following exposure to all except the Oldsmobile sample in the absence of activation indicates that these samples contain one or more components that are direct-acting chromosome-damaging agents. Although the significance of the differences among these diesel and gasoline emission samples cannot be inferred from the data, this test gave the following qualitative comparative potency ranking: Nissan > Rabbit, Mustang >> Caterpillar, Oldsmobile (Mitchell et al., 1981).

Two assays, one measuring morphologic transformation in 3T3 cells and the other measuring viral enhancement of transformation in SHE cells, were used to observe the effects of gasoline and diesel emissions on oncogenic transformation. As with the 3T3 mutation assay, dose-related responses in transformation frequency in 3T3 cells were not observed for any of the samples (Caterpillar, Nissan, Oldsmobile, and Mustang). Both the Nissan and Mustang samples induced a significant number of transformed foci in the absence of metabolic activation while only emissions from the Mustang had a transformation frequency significantly greater than that of controls in the presence of metabolic activation (Curren et al., 1981).

In the viral enhancement assay, the Nissan appeared to be the most potent followed by the Rabbit and Mustang, which were equipotent, and the Oldsmobile and Caterpillar according to the lowest effective concentration tested that induced significant enhancement (Casto et al., 1981). However, if the data from three separate experiments were combined to determine the slope of the pooled dose-response curve for each sample, the comparative potency ranking would be: Nissan, Mustang > Rabbit > Oldsmobile (Caterpillar is considered negative). Because the variation in response between the

three experiments was significant (r^2 value as low as 0.18), each experiment was analyzed separately and the experiment resulting in the highest r^2 was used to determine the following potency ranking: Nissan > Rabbit > Oldsmobile, Mustang (Lewtas, 1983). Despite these variations, the ranking for light-duty diesel engine samples remains fairly constant: Nissan > Rabbit > Oldsmobile.

In the skin tumor initiation assay in SENCAR mice, the four diesel samples varied significantly in the tumorigenic responses they produced, ranging in activity from 0 to 5.7 papillomas/mouse (Nesnow et al., 1982). Papillomas were produced in all samples except for the Caterpillar. Excess tumor multiplicity activities in papillomas per mouse at 1 mg of extract were calculated as follows: Nissan - 0.59, Oldsmobile - 0.31, Rabbit - 0.24, and Mustang - 0.17 (Albert et al., 1983). These data indicate that only the Nissan extract can be considered a strong tumor initiator, with activity similar to that of roofing tar.

A comparison of these test systems reveals that, in general, there is a consistency in the comparative potency of these extracts with the Nissan sample the most active and the Caterpillar sample the least potent in all bioassays. The main issue, however, is whether these data demonstrate sufficient biological similarity among the different samples to warrant their use in predicting the effects of other diesel mixtures. Based on these data, results from heavy-duty diesel engine (Caterpillar) emissions would severely underestimate the effects of a light-duty diesel engine and should not be used for that purpose. Within the light duty class of diesel engines, there appears to be reasonably close agreement between the Oldsmobile and Rabbit engines while the Nissan is considerably more potent. Because of the Nissan data, it would not be prudent to assume that all

light-duty diesel engine emissions are sufficiently similar as to their biological effects.

The available information on components of the four diesel and one gasoline emission samples indicates a wide range of organic extractable material (Table B-2). Benzo(a)pyrene (BaP) content per mg extract also varied considerably, from 0.0002 to 0.11% (Lewtas et al., 1981). Nesnow et al. (1982) state that the tumor data from the SENCAR mouse skin tumor initiation assay cannot be explained solely by BaP content since there is no significant relationship between tumor incidence and BaP content in each complex mixture (including diesel and gasoline engine, roofing tar, and coke oven emissions). They estimate that BaP accounts for only 20-30% of the activity seen and that other constituents must be contributing toward the tumorigenic activity. Whether this contribution is through interaction or direct component activity cannot be determined from the available data.

It is evident that the available component data does not meet the sufficient similarity criteria at least in the case of BaP content for the Nissan and Mustang samples. If BaP can account for no more than 30% of the tumorigenic activity of the mixture, it is apparent that additional component information is necessary before the issue of sufficient constituent similarity can be accurately evaluated.

TABLE B-2

Results of Extraction and Benzo(a)pyrene Analysis*

Sample Source		Extractable Matter percent	Benzo(a)pyrene ng BaP / mg extract	Benzo(a)pyrene ng BaP / mg particulate
Diesel	CAT	26-27	2	0.5
	NISSAN	4-8	1173	96.2
	OLDS	12-17	2	0.4
	VW RAB	18	26	4.6
Gasoline	MUSTANG	39-43	103	44.1

*Source: Lewtas et al., 1981

APPENDIX C

ANALYSIS OF THE SAMPLE STUDIES FROM THE INTERACTION DATA BASE

Thirty-two studies were selected from the U.S. EPA interaction data base (see Appendix A) for detailed evaluation of the statistical methods that were employed in determining the type of toxic interaction. The evaluation included the appropriateness of the statistical method used and the correctness of interpretation of the statistical results. The 10% random sample was stratified by the type of statistics used. The following text is the evaluation of each study.

Carlson (1973) pretreated rats with either phenobarbital (PB), 3-methylcholanthrene (3-MC), saline or corn oil vehicle, then exposed them to air, 1,1,1-trichloroethane or 1,1,2-trichloroethane. Endpoints assessed were liver and body weights, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and liver glucose-6-phosphatase. Analysis was by 2-way analysis of variance to assess differences between pretreatments, inhalation treatments and the interactions between the two. The analysis was appropriate. No differences were found in liver or body weights, and it was concluded that 3-MC did not potentiate the hepatotoxicity of the trichloroethanes, but PB did and enhancement was greater with 1,1,2-trichloroethane than with 1,1,1-trichloroethane.

Short et al. (1977) exposed mice and rats to continuously inhaled air or 1,1-dichloroethylene (VDC), then to one of disulfiram, diethyldithiocarbamide (DDC), thiram, apteine, methionine, N-acetylcysteine, SKF 525-A, cobaltous chloride, phenoxybenzamine, propanolol, Vitamin C or to Vitamin E. Endpoints assessed were death, organ damage as assessed by serum enzymes and histopathology, changes in liver and kidney, and radioactivity in protein. Statistical methods used were calculation of the LC_{50} and LT_{50} for VDC for assessment of survival, and the 2 sample rank test and Fisher's exact test for the other endpoints. No methods were used to control for multiple comparisons. Survival analysis would have been more appropriate to use. No

negative control group was present. Repeated measures analysis should have been used to assess the changes across time in SGOT and SGPT. They concluded that disulfiram reduced the severity of the lethal, hepatotoxic and renal effects of VDC in mice, that DDC and thiram protect mice from the lethal effects of VDC, and that the dithiocarbamates protected against the toxicity of VDC.

Castro et al. (1974) exposed rats to SKF 525-A, Sch 5705, Sch 5706, Sch 5712, CFT 1201, Lilly 18947, DPEA, promethizine or vehicle control, then to CCl_4 or olive oil vehicle. Endpoints assessed were ethylmorphine N-demethylase activity (EM-ase), cytochrome P-450 activity, peroxidation of liver microsomal lipids, CCl_4 concentration in liver, protein concentration, and NADP-linked isocitric dehydrogenase (ICD) activity in plasma. In examination of the time course of CCl_4 concentration, they used Student's t-test and the Mann-Whitney-U test for comparison at each time point. In this situation the groups were CCl_4 alone vs. CCl_4 and other compound, sometimes varying the level of CCl_4. To examine the effects on EM-ase, ICD and P-450, they made comparisons via 2-way ANOVA. To examine the time course of lipid peroxidation and body temperature, they used a 2-way ANOVA at each time point, whereas a repeated measures analysis would have been correct. They concluded that "although these compounds tested are known to inhibit cytochrome P-450 dependent drug-metabolizing enzymes in liver microsomes, they apparently do not evoke their protective effects by slowing the elimination of CCl_4." These conclusions are appropriate in light of the methods used.

Andrews et al. (1977) examined the effects of toluene on metabolism, disposition and hemopoietic toxicity of [H3]benzene, particularly red cell ^{59}Fe incorporation as a measure of erythropoiesis. Two 2x2 factorial

experiments were conducted on mice, with the administration of 0 or 880 mg/kg benzene and 0 or 1720 mg/kg toluene as one experiment, and the administration of 0 or 440 mg/kg benzene and 0 or 1720 mg/kg toluene as the other. Endpoints assessed were benzene metabolites in urine, expressed as percent administered dose and as benzene equivalents, percent ^{59}Fe utilization, exhaled [3H]benzene, and levels of [3H]benzene in liver, spleen, epididymal fat pads, blood, and bone marrow. Although the experiments were conducted as 2x2 factorials, making the use of 2-way ANOVA appropriate, Student's t-tests were in fact used. Furthermore, the time course of accumulation of [3H]benzene in tissues was analyzed by t-tests at each time point, whereas repeated measures analyses were appropriate. The authors concluded that toluene reduced the level of urinary metabolites of benzene and also reduced the benzene-induced inhibition of erythrocyte ^{59}Fe uptake. These conclusions are consistent with the results of the statistical methods used, but may not be valid due to the increased likelihood of false positives with these methods.

Friedman and Eaton (1978) studied the effects of an inhibitor of mixed function oxidase (MFO) activity, piperonyl butoxide (PB), on methylmercury (MM) toxicity. Rats were fed diets containing either 0, 20 or 40 ppm methylmercury, and either 0, 0.5 or 1.0% PB. Endpoints assessed were weight gain and mortality. No statistical methods and no dose-response models were used. The authors conclude that "PB synergises MM poisoning in a dose-dependent fashion."

Biancifiori et al. (1967) examined the effects of estrogen on the pathway through which chemical carcinogens exert their action. Both ovariectomized and intact mice were given estrone at 0, 500 or 1000 µg/ℓ drinking water, and the mice were administered either nothing, or one of

9,10-dimethyl-1,2-benzanthracene (DMBA), 1,2:5,6-dibenzanthracene (DBA), 20-methylcholanthrene (MC) or 3,4-benzopyrene (BP) at 0.5% in almond oil, twice weekly until 8 weeks of age. Endpoints assessed were mammary carcinoma, survival, gastric tumor, ovarian tumor, leukemia, and lung tumor. Although no statistics were used, the authors conclude that "administration of oestrone increased the incidence of mammary carcinomas in both intact and ovariectomised mice when DBA or MC were the carcinogens; only a minimal effect was obtained with BP and the result with DMBA was equivocal," "squamous carcinomas of the forestomach occurred when the carcinogen was BP or DMBA," and "DBA with oestrone induced ovarian tumours."

Cone and Nettesheim (1973) investigated the effects of high levels of vitamin A on the toxicity of 3-methylcholanthrene (MCA) in the respiratory tract epithelium of the rat. All animals received vitamin A, either in doses of 17, 87 or 1740 µg/week, and either 0 or 5 mg MCA. The endpoint assessed was respiratory tract tumor. Although no statistics were given, the authors concluded that vitamin A has an inhibitory effect on the development of respiratory tract tumors.

Daoud and Griffin (1980) investigated the effect of retinoic acid, butylated hydroxytoluene (BHT), selenium (Se) and sorbic acid on azo-dye hepatocarcinogenesis in the rat. All animals received a diet containing 0.05% 3'-methyl-4-dimethylaminoazobenzene (3'-MeDAB), and either nothing, 0.05% BHT, 1 or 2% sorbic acid, 0.02% retinoic acid or 2 or 4 ppm Se, but no combinations or the latter four compounds. The endpoint assessed was liver carcinoma. Although no statistics were given, the authors conclude that protection was "afforded the animals given the azo compound by the dietary supplementation with either retinoic acid or BHT."

Schlede et al. (1970) examined the stimulatory effect of benzo(a)pyrene (BaP) and phenobarbital pretreatment on the biliary excretion of BaP metabolites in the rat. Animals were pretreated with either BaP, phenobarbital or vehicle, then received either 10 or 300 μg of ^{14}C-labeled BaP (BaP-^{14}C). The endpoint assessed was the rate of excretion of metabolites of BaP-^{14}C into bile. Although no statistics were given, the authors conclude that "pretreatment of rats with BaP or phenobarbital prior to the i.v. injection of 10 or 300 μg of BaP-^{14}C enhances the rate of excretion of metabolites of BaP-^{14}C into the bile."

Ito et al. (1973) examined the effect of polychlorinated biphenyls (PCBs) on tumorigenesis by benzene hexachloride (BHC) in mouse liver. Animals received diets containing 0, 100, 250 or 500 ppm PCBs, alone or in conjunction with 0, 100 or 250 ppm of α, β or γ-BHC. Endpoints assessed were histopathology of the liver, liver weight and body weight. No statistics were given. The authors conclude that "PCBs themselves induced hepatic neoplasms in mice and also promoted the induction of tumors by α-BHC and β-BHC."

Magos et al. (1974) describe the effect of cadmium pretreatment on the nephrotoxic action and kidney uptake of mercury in male and female rats. Animals were pretreated with either 0 or 2x2.46 mg/kg $CdCl_2$, then treated with 0, 0.5, 1.0 or 1.5 mg/kg $HgCl_2$. Endpoints assessed were μg Hg^{2+} in kidneys/100 g bw, and severity of tubule damage. No statistics were given. The authors state that there was a "significant sex difference observed in effect of Cd^{2+} pretreatment on the uptake of Hg^{2+} by the kidneys" and a "protective effect of Cd pretreatment against tubular damage caused by mercury."

Moxon and DuBois (1939) investigated the influence of arsenic and other elements on the toxicity of seleniferous grains in the rat. Twelve groups of animals were given diets containing seleniferous wheat and 11 of these groups were given 5 ppm of one of the following elements in drinking water: W, F, Mo, As, Cr, V, Cd, Zn, Co, U, Ni. A thirteenth group received a diet containing selenium-free wheat. The elements in drinking water were not given in conjunction with a diet containing selenium-free wheat. Endpoints assessed were survival and Se content in liver. No statistics were given. A further experiment was then conducted since it appeared from the initial experiment that "tungsten and arsenic, especially the latter, reduced selenium toxicity in some manner." Animals were fed diets containing either selenium-free or seleniferous wheat, then drinking water containing either nothing, 2.5 ppm W or 2.5 ppm As. Endpoints assessed were survival and liver damage, and again no statistics were given. The authors concluded that F, Mo, Cr, Cd, Zn, Co, Ni and U given with Se caused an increase in mortality, that W seemed to reduce the mortality rate of rats with Se, and As prevented Se poisoning symptoms, although it did not prevent liver damage appreciably.

Thind and Biery (1974) investigated the antagonism of renal angiographic effects of cadmium by zinc in the dog. In one group, animals received intrarenal doses of cadmium acetate (2-20 mg) and zinc chloride (2-20 mg). The animals in a second group received a series of arteriograms for the following exposure sequence: control, vasoactive drug (angiotensin, epinephrine, norepinephrine), cadmium acetate + vasoactive drug, vasoactive drug, zinc chloride + cadmium acetate + vasoactive drug; this sequence was then repeated in each dog with two different vasoactive drugs. No quantitative data were presented; instead, the authors presented the radiographs from the angiograms. The authors conclude that "pretreatment of the

renal vasculature with zinc ions in the present study effectively blocked the acute inhibitory effects of cadmium ions in the kidney of normal dogs."

Drew and Fouts (1974) studied the effects of pretreatment with either phenobarbital (PB), 3-methylcholanthrene (3-MC), or chlorpromazine (CPZ), on p-xylene toxicity in rats. Animals were subsequently either exposed to p-xylene vapors or were injected with p-xylene. The authors allude to the presence of control groups, but the nature of these groups is not stated. The LD_{50} for injected p-xylene and the LC_{50} for inhaled p-xylene were calculated for the control groups and the pretreatment groups. This work was presented in abstract. No data were given, and the statistical methods used were not specified. The authors conclude that the pretreatments raise the LC_{50} of inhaled p-xylene, whereas only 3-MC increases the LD_{50} of injected p-xylene.

Dietz (1980) investigated the roles of 2-butanol, 2-butanone and 2,3-butanediol in potentiating CCl_4 hepatotoxicity. Rats were pretreated with various dosages (unspecified) of one of these three compounds, then were administered one dose of CCl_4. In addition, some animals were pretreated with pyrazole. Endpoints assessed were SGPT, glucose-6-phosphatase activity and triglyceride concentration. This work was presented in abstract form. No data were given, and the statistical methods used were not specified. The authors concluded that the capability of 2-butanol and 2-butanone to potentiate CCl_4 hepatotoxicity is due to their further metabolism to 3-hydroxy-2-butanone and 2,3-butanediol.

Bhargava and Way (1974) examined the effect of 1-phenyl-3-(2-thiazolyl)-2-thiourea (PTT) on morphine analgesia, tolerance and physical dependence in the mouse. Animals previously rendered tolerant to morphine were pretreated with PTT or vehicle, and brain uptake of norepinephrine, dopamine, copper,

serotonin, acetylcholine, choline and morphine were assessed. The statistical methods used were not specified. The authors state that the analgesic effect of morphine was potentiated by PTT, but this effect was not correlated with changes in brain levels of norepinephrine, dopamine, copper, serotonin, acetylcholine and choline.

Gupta and Gupta (1977) investigated the effect of the insecticide endosulfan on pentobarbitone sleep time and concentration of pentobarbitone in blood and brain in rats. Animals received 0, 1, 2.5 or 5 mg/kg endosulfan for 7 or 15 days, then all animals received 50 mg/kg pentobarbitone. The statistical methods used were not specified. The higher doses of endosulfan (2.5 and 5 mg/kg) were associated with increased induction time and decreased sleep time. There was no change in pentobarbitone concentrations in blood and brain.

Gunn et al. (1968) studied compounds that potentially were protective against cadmium toxicity. Mice received $CdCl_2$ (doses ranged between 0.0055 and 0.0664 mM/kg) in conjunction with control, an amino acid (either alanine, arginine, asparagine, cysteine, glycine, isoleucine, lysine, methionine, proline, serine, threonine, valine, leucine or phenylalanine), 2,3-dimercaptopropanol (BAL), selenium dioxide or zinc acetate. Endpoints were death at 7 days, and percent Cd uptake in various organs (testes, kidneys, heart, lungs, pancreas, spleen and gastrointestinal tract). The statistical methods used were not specified. The authors found that cadmium destroyed the testis, and, while this destruction was prevented by cysteine, that lethality was increased by cysteine. Moreover, BAL, selenium and zinc also protected the testis from cadmium, but did not affect levels of kidney cadmium nor toxicity of cadmium. Furthermore, none of the other amino acids was protective against cadmium damage of the testis or increased cadmium toxicity.

Jernigan and Harbison (1982) investigated the role of 2,5-hexanedione (2,5-HD) in halocarbon hepatotoxicity. Mice were pretreated with corn oil, 2,5-HD or phenobarbital sodium (PB), then subsequently received one of the following halocarbons: $CDCl_3$, $CHCl_3$, CCl_4, trichloroethylene (TCE), 1,1,2-trichloroethane (TRI), or perchloroethylene (PERC). Endpoints assessed were hepatic cytochrome P-450, NADPH cytochrome c reductase, aniline hydroxylation, p-nitroanisole O-demethylation and aminopyrine N-demethylation, and serum alanine amino transferase activity. Statistical comparisons were made using analysis of variance. Because of the absence of a control group for the halocarbons, and the authors' continual testing by comparison back to the corn oil pretreatment group, this paper is insufficient to assess the potentiation of any of these halocarbons by 2,5-HD. The authors conclude that ketone potentiation of $CHCl_3$-induced hepatotoxicity was demonstrated in mice and that pretreatment with 2,5-HD can potentiate the hepatotoxicity of other halocarbons.

Snyder et al. (1981) studied the effect of ethanol ingestion on hematotoxicity of inhaled benzene in mice. The inhalation-ingestion groups were as follows: air + water, air + 5% ethanol, air + 15% ethanol, 300 ppm benzene + water, 300 ppm benzene + 5% ethanol, 300 ppm benzene + 15% ethanol. Endpoints assessed were body weight and blood counts. The statistical methods used were not stated. The authors find that the "results indicate a true potentiation of the toxic effects of benzene by ethanol."

Csallany and Ayaz (1978b) assessed the effects of NO_2 and vitamin E in mice. Animals were exposed first to either filtered air or 0.5 ppm NO_2 or 1.0 ppm NO_2, then to 0, 30 or 300 ppm vitamin E or 30 ppm N,N'-diphenyl-p-phenylenediamine (DPPD). Endpoints studied were body weight, tissue weights, lipofuscin pigment (LFP) concentration in tissues, and survival

rates. The statistical methods used were not specified. The authors conclude that continuous low level NO_2 exposures do not result in higher concentrations of tissue organic solvent soluble LFP, but NO_2 does have an overall detrimental effect on animals, as measured by lowered whole-body weights and survival rates.

El-hawari (1978) examined the potentiation of dibromoethane (EDB) toxicity by disulfiram (DS), thiram, diethyldithiocarbamate and carbon disulfide in mice. Animals were pretreated with either DS, thiram, diethyldithiocarbamate or CS_2, then treated with EDB. Only one dose of the various pretreatment compounds was administered. It was unclear what control groups were present. Endpoints assessed were SGPT, SGOT, blood urea nitrogen (BUN) levels and survival. The report is in abstract form. No statistical methods are stated. The authors conclude that pretreatment with any of these compounds enhances EDB toxicity.

Agarwal et al. (1983) studied the interactions of CBr_4 and chlordecone (CD) in rats. Animals were fed diets containing either 0 or 10 ppm CD, then were injected with either 0, 25, 50, 100 or 125 µℓ CBr_4. Endpoints assessed were urine parameters, including volume, osmolality, blood, protein, glucose; p-aminohippurate (PAH) and tetraethylammonium (TEA) levels in the renal cortex; SGOT and SGPT levels. Since the authors felt that CBr_4 was acting like a nephrotoxin, a second experiment was undertaken in which animals were fed diets containing either 0 or 10 ppm CD, then were administered either vehicle or 54 µℓ CCl_4 or 75, 125 or 175 mg CBr_4. Endpoints measured were PAH and TEA levels in renal cortical slices. In both experiments, statistical methods were not specified, although comparisons were made to control groups. The authors concluded that chlordecone did not modify renal slice response, and that CD does not potentiate CBr_4 hepatotoxicity.

Berlin and Lewander (1965) investigated the effect of 2,3-dimercaptopropanol (BAL) on brain uptake of mercury in mice given mercuric chloride. In the acute experiment, animals were given 0.5 mg/kg Hg, then either 0 or 0.3 mg/kg BAL. Endpoints measured were tissue Hg concentration. In the chronic experiment, animals were given 1 mg/kg $^{203}HgCl_2$ and either 0 or 2 mg/kg bw BAL for 16 days. Again, the endpoints assessed were Hg concentrations in tissue. There were no negative control groups (no BAL, no mercuric chloride). The statistical methods used were not specified. The authors concluded that BAL does not affect Hg elimination.

deFerreyra et al. (1983) assessed the potentiation of CCl_4 necrosis by cysteine and tryptophan, both alone and together, in the rat. Experimental groups were as follows: control, CCl_4, cysteine, tryptophan, cysteine + CCl_4, tryptophan + CCl_4, and cysteine + tryptophan + CCl_4. Endpoints measured were ICD levels and degree of liver necrosis. Although the authors state that a 2-way analysis of variance was used, it is unclear what groups were compared. All other comparisons were to the control group, using unspecified statistical methods. This experiment is missing one experimental group (cysteine + tryptophan); otherwise a 3-way analysis of variance would have been the correct procedure. The authors conclude that administration of cysteine but not tryptophan decreased ICD, and when both cysteine and tryptophan were given together, a "marked protective effect is observed."

Agarwal and Mehendale (1984) studied the potentiation of CCl_4 hepatotoxicity by chlordecone (CD) in ovariectomized rats. Animals were either sham operated or ovariectomized, then fed diets containing either 0. or 10 ppm CD, then received either 25 µℓ CCl_4 or vehicle. Endpoints measured were SGPT, SGOT, isocitric dehydrogenase (ICD) and ornithine carbamyl transferase (OCT) activity. The authors used student's t-test and one-way

analysis of variance, making comparisons to controls, although 3-way analysis of variance would have been the correct method to investigate the interplay of these compounds. They also measured biliary excretion of phenolphthalein glucuronide (PG) over time, which would have been correctly analyzed by a repeated measures analysis. The authors conclude that "CD induced potentiation of CCl_4 hepatotoxicity in ovariectomized rats was not significantly enhanced as compared to earlier observations in intact females."

Kinnamon and Bunce (1965) examined the effects of copper, molybdenum and zinc on ^{65}Zn tissue distribution and excretion in the rat. There were eight experimental groups consisting of the combinations of 0 or 100 mg/kg Cu, 0 or 800 mg/kg Mo and 0 or 5000 mg/kg Zn in feed. Endpoints assessed were body weight, weight gain, feed consumption, percent Zn retention in tissues and percent Zn excretion in urine. Comparisons were made to controls, using t-tests, although a 3-way analysis of variance would have been the correct procedure. The authors concluded that "Zn, not Mo or Cu, significantly influences tissue distribution and excretion of tracer Zn."

Jaeger and Murphy (1973) studied the effects of 1,1-dichloroethylene (1,1-DCE), corticosterone or acrolein on barbituate action in the rat. Animals were pretreated with either 0 or 400 mg/kg 1,1-DCE, then were given either pentobarbital (PB) or hexobarbital (HB). Endpoints assessed were sleep time, barbituate concentration in serum and brain, and serum corticosterone concentration. A second and third experiment were conducted in which pretreatment was either 0 or 25 mg/kg corticosterone or 3 mg/kg acrolein, assessing the same endpoints. Statistical techniques employed included t-test, analysis of variance and regression, although it is impossible to tell which technique (t-test or analysis of variance) was used to make certain comparisons. There was no negative (untreated) control in any

experiment. The authors concluded that both 1,1-DCE and corticosterone alter PB-induced, but not HB-induced, sleep time, and that acrolein increases both PB and HB sleep time.

Hasumura et al. (1974) investigated the effect of chronic ethanol consumption on CCl_4 hepatotoxicity in the rat. Experimental animals were fed diets consisting of ethanol (36% of total calories), and control animals were pair-fed diets in which ethanol had been isocalorically replaced by carbohydrate. Animals then received either 0.5 mℓ/kg CCl_4 or mineral oil. Endpoints measured were serum ornithine carbamyl transferase (SOCT), SGPT, bilirubin, total lipid, cytochrome P-450, aminopyrine N-demethylase activity, and glucose-6-phosphatase activity. Paired Student's t-test was used to compare ethanol to pair-fed control, whereas a randomized complete block design would have been the correct procedure to make this comparison, allowing for multiple comparisons. The authors concluded that "chronic ethanol administration to rats potentiates CCl_4 hepatotoxicity," although they did not use methods that would allow for the assessment of interactions.

Harbison and Becker (1971) examined the effect of treatment with phenobarbital (PB) or SKF 525A on diphenylhydantoin (DPH) disposition on pregnant mice. All animals received 100 mg/kg DPH, after pretreatment with either control, PB or SKF 525A. Endpoints measured were DPH metabolism in plasma, placenta, fetus, amniotic fluid, liver, brain, fat, and muscle over time. They used Student's t-test to compare each pretreatment group with DPH alone at each time point, although a repeated measures analysis would have been the correct procedure. They concluded that pretreatment with PB enhanced the metabolism of DPH, with decreased plasma DPH and DPH-induced teratogenicity and *in utero* deaths, while pretreatment with SKF 525A decreased metabolism of DPH, with increased plasma DPH and DPH-induced teratogenicity and *in utero* deaths.

Csallany and Ayaz (1978a) investigated the effects of intermittent NO_2 exposure and vitamin E in rats. Animals were fed either vitamin E deficient (0 ppm), normal (30 ppm), or high (300 ppm) diets, then were exposed to either air or 15 ppm NO_2 for either 5 or 18 weeks. Endpoints assessed included methemoglobin levels, histopathology, lipofuscin pigment concentration in tissue and fatty acid component in lung tissue. Student's t-test was used to make comparisons between the treatment groups, although it was not always clear which groups were being compared. Analysis of variance techniques would have been correct. The authors concluded that "intermittent NO_2 exposure, under the described conditions, did not cause ultimate changes of the biochemical parameters measured."

Derr et al. (1970) examined the synergism between cobalt and ethanol on rat growth rate. Water, allowed ad libitum, was replaced with either 0 or 10% ethanol, and either 0 or 1 mg Co/10 mℓ H_2O. Endpoints measured were body weight, hematocrit, heart weight, heart Zn, and heart-to-body weight ratio. Student's t-test was used to compare the various groups, and an additive model was used to calculate an expected body weight for the ethanol + Co group, which was then compared with the observed body weight for that group by Student's t-test. A two-way analysis of variance would have been the correct procedure. The additive model that was used added the weight deviations from the control in order to predict the weight deviation of the two chemicals combined. The authors did not provide any biological justificaton for such a model. Even a simple method using relative potencies would be better justified. The authors' conclusion was that ethanol and cobalt have a synergistic effect.

APPENDIX D

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