APPENDIX A
A GENERAL REFERENCE ON PHYSIOLOGICALLY-BASED PHARMACOKINETIC/PHARMACODYNAMIC (PBPK/PD) MODELING

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PHYSIOLOGICALLY BASED PHARMACOKINETIC AND PHARMACODYNAMIC MODELING

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INTRODUCTION

At first glance, the topic of physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling appears to be outside the central theme of this book. However, with the advances of modern biology and computational technology, it is just a matter of time for any area of biomedical sciences to be integrated with computer science. Two aspects are particularly important for the application of PBPK/PD modeling in cancer research, thus underscoring the relevance for the inclusion of this chapter in the book: (1) incorporation of PBPK/PD modeling in any biomedical research can avoid•Q2 unnecessary experiments, thereby conserving precious resources, and (2) computer simulations (i.e., in silico experimentation) using validated PBPK/PD models will minimize animal usage.

The intent of this chapter is to (1) introduce the general concept and background knowledge of PBPK/PD modeling; (2) provide some examples of application of PBPK/PD modeling; (3) illustrate the utility of PBPK/PD modeling, particularly in the pharmaceutical drug development process; and (4) project future development on “second-generation” PBPK/PD modeling and In silico toxicology. An emphasis was given to introducing the concepts of PBPK/PD modeling rather than the details of its techniques and processes. For more detailed background and conceptual information on PBPK modeling, the readers are referred to our earlier introductory discussion (Yang and Andersen, 1994) and the two “timeless papers” by Bischoff and Brown (1966) and Dedrick (1973). For more extensive information, particularly regarding modeling techniques, the reader should consult the two future books from our laboratories (Reddy et al.,• 2004; Yang et al., 2004).

WHAT IS PHYSIOLOGICALLY BASED PHARMACOKINETICS? WHAT ARE THE DIFFERENCES BETWEEN PBPK AND CLASSICAL PHARMACOKINETICS?

Physiologically based pharmacokinetics (PBPK), as the name implies, is a special branch of pharmacokinetics

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where physiology and anatomy of the animal or human body as well as the biochemistry of the chemical or chemicals of interest are incorporated into the conceptual model for computer simulation. *Classical pharmacokinetics* refer to those empirical noncompartmental or compartmental pharmacokinetic studies routinely practiced in the pharmaceutical industry (van de Waterbeemd and Gifford, 2003). As will be illustrated later, the compartments of a PBPK model have anatomic and physiologic significance. This is a major difference from empirical noncompartmental or compartmental pharmacokinetic modeling approaches. PBPK models can be used to describe concentration–time profiles in individual tissue/organ and in the plasma or blood. When the concentration of a certain target tissue, rather than the plasma concentration, is highly related to a compound's efficacy or toxicity, PBPK modeling will be a more useful tool than classical pharmacokinetic models for describing PBPK/PD relationships and thus make a better prediction of the time course of drug effects resulting from a certain dose regimen for the compound of interest. Furthermore, PBPK models in combination with absorption simulation and quantitative structure–activity relationship (QSAR) approaches will bring us closer to a full prediction of drug disposition for pharmaceutical new entities and help streamline the selection of lead drug candidates in the drug discovery process (van de Waterbeemd and Gifford, 2003). Lastly, unlike empirical noncompartmental and compartmental pharmacokinetics, PBPK modeling is a powerful tool for extrapolation, be it for interspecies, interroutes, interdoses, interlife stages, and so on.

The concept of PBPK had its embryonic development in the 1920s and 1940s; for a more detailed early history, readers are referred to two books which are in preparation (Reddy et al., 2004; Yang et al., 2004). PBPK modeling blossomed and flourished in the late 1960s and early 1970s in the chemotherapeutic area mainly due to the efforts of investigators with expertise in chemical engineering process design and control. Two notable pioneers in this development are Kenneth B. Bischoff, then at the University of Texas, Austin, Texas, and Robert Dedrick of Biomedical Engineering and Instrumentation Branch, Division of Research Services, National Institutes of Health, Bethesda, Mariland. Two timeless publications by these investigators are, respectively, "Drug Distribution in Mammals" (Bischoff and Brown, 1966) and "Animal Scale-Up" (Dedrick, 1973); these articles are highly recommended to those who are interested in PBPK modeling. In the mid-1980s, two articles on PBPK modeling of styrene and methylene chloride (Andersen et al., 1987; Ramsey and Andersen, 1984) started yet another "revolution" in the toxicology and risk assessment arena. Today, there are more than 700 publications directly related to PBPK modeling on industrial chemicals, drugs, environmental pollutants, and simple and complex chemical mixtures (Reddy et al., 2004).

A PBPK model, graphically illustrated in Figure 23.1, reflects the incorporation of basic physiology and anatomy. The compartments actually correspond to anatomic entities such as liver, lung, and so on, and the blood circulation conforms to the basic mammalian physiology. In this specific model, a published example on methylene chloride, it is quite obvious that the exposure route of interest is inhalation because the lung and gas exchange compartments are prominently displayed with intake (CI) and exhalation (CX) vapor concentrations indicated. Oral and/or dermal exposures may be added easily to the gastrointestinal tract compartment or general venous circulation, respectively. Some tissues (e.g., richly or slowly perfused tissues in Fig. 23.1) are "lumped" together because there is insufficient evidence to conclude that each of these tissues is kinetically distinct enough for the specific chemical to warrant a separate compartment.

If one draws an analogy of the "scale-up" from a laboratory chemical engineering process to a chemical plant to the scale-up of a mouse to a human, one finds that both situations are governed by a great number of physical and chemical processes. In mammals, the physical processes (i.e., mass balance, thermodynamics, transport, and flow) often vary in a predictable way. However, chemical processes such as metabolic reactions may vary greatly and are less predictable among species. These physical and chemical processes interact in the body such that the pharmacokinetics of any given chemical between one species and another may be more (or less) predictable depending on the amount of background information available.

HOW DOES A PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL WORK?

A PBPK model applies fundamental physiologic, biochemical, and engineering principles to describe the distribution and disposition of chemicals in the body at any given time. The process and approach may be summarized in a flowchart (Fig. 23.2). Once the chemical of interest and the problems needing to be addressed are identified, a thorough literature evaluation is conducted.

The fundamentals of PBPK modeling are to identify the principal organs or tissues involved in the disposition of the chemical of interest and to correlate the chemical absorption, distribution, metabolism, and excretion within and among these organs and tissues in an integrated and biologically plausible manner. A scheme is usually formed where the normal physiology is followed in a graphical manner (i.e., a conceptual model as in Fig. 23.1). Within the boundary of the identified compartment (e.g.,

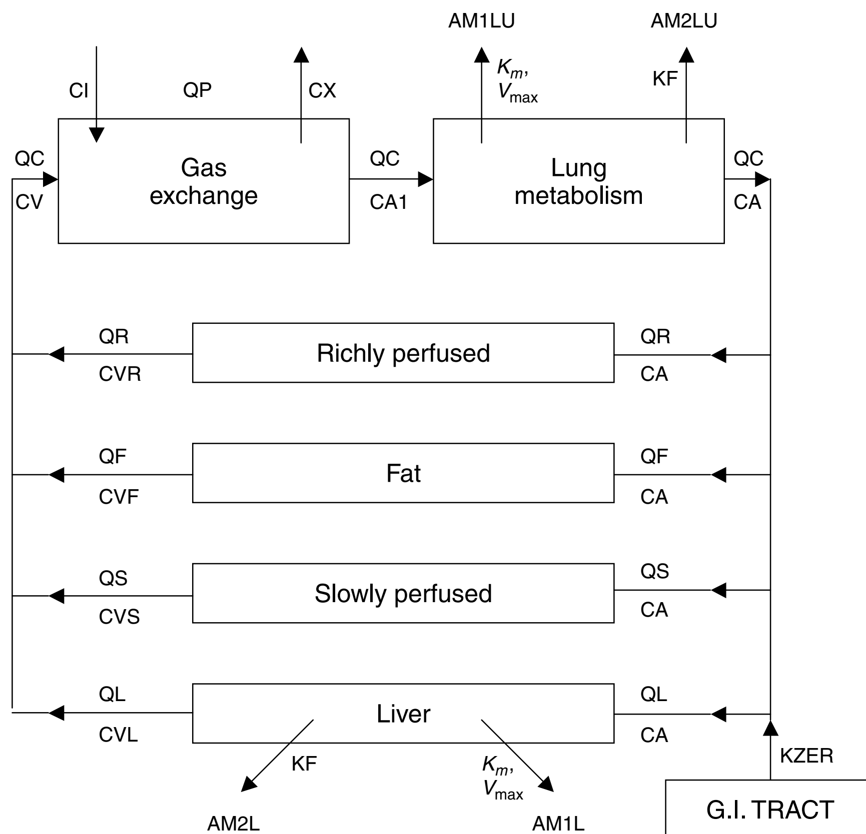

Figure 23.1. Graphical representation of a PBPK model for methylene chloride. (Andersen et al., 1987).

an organ or tissue or a group of organs or tissues), whatever "comes" in must be accounted for via whatever "goes out" or whatever is transformed into something else. This "mass balance" is expressed as a mathematical equation with appropriate parameters carrying biologic significance. A series of such equations representing all of the interlinked compartments are formulated to express a mathematical representation, or model, of the biologic system. This model can then be used for computer simulation to predict the time course behavior of any given parameter. Three sets of parameters are needed for PBPK model building: physiologic parameters (e.g., ventilation rates, cardiac output, organs as percent body weight), thermodynamic parameters (e.g., tissue partition coefficients, flow rates), and biochemical parameters (e.g., K_m and V_{max}). Most, if not all, of the parameters for laboratory animals are available in relevant literature, such as the *Biological Data Book* and the special report by the International Life Sciences Institute (ILSI) on the compilation of physiologic parameters for PBPK models (Brown et al., 1997). When information gaps exist, the solution is either an empirical one via experimentation or through allometric extrapolation, usually based on a power function of the body weight (Lindstedt, 1987).

The U.S. Environmental Protection Agency (EPA) guidance document (2002) includes a very nice discussion on "modeling the data." Although it is not necessarily for PBPK modeling, the discussion reflects some "dos" and "don'ts" on computer modeling. We quote some of the passages below:

> The• selection of a mathematical model structure to fit the data being analyzed should be guided by the biology of the common mechanism of toxicity, the toxicokinetics of the chemicals, and the observed shapes of their dose-response curves and the experimental designs used to generate the data. If available, pharmacodynamic and pharmacokinetic data should be considered in order to account for tissue concentrations and to aid in defining dose-response relationships across different species, routes and time-frames of exposure . . . .
>
> . . . Although it is not possible to recommend the use of specific models, a few points that should be considered in modeling the data follow:
>
> - Modeling of individual animal data is desirable; however, if this is not practical, then use

• Q3

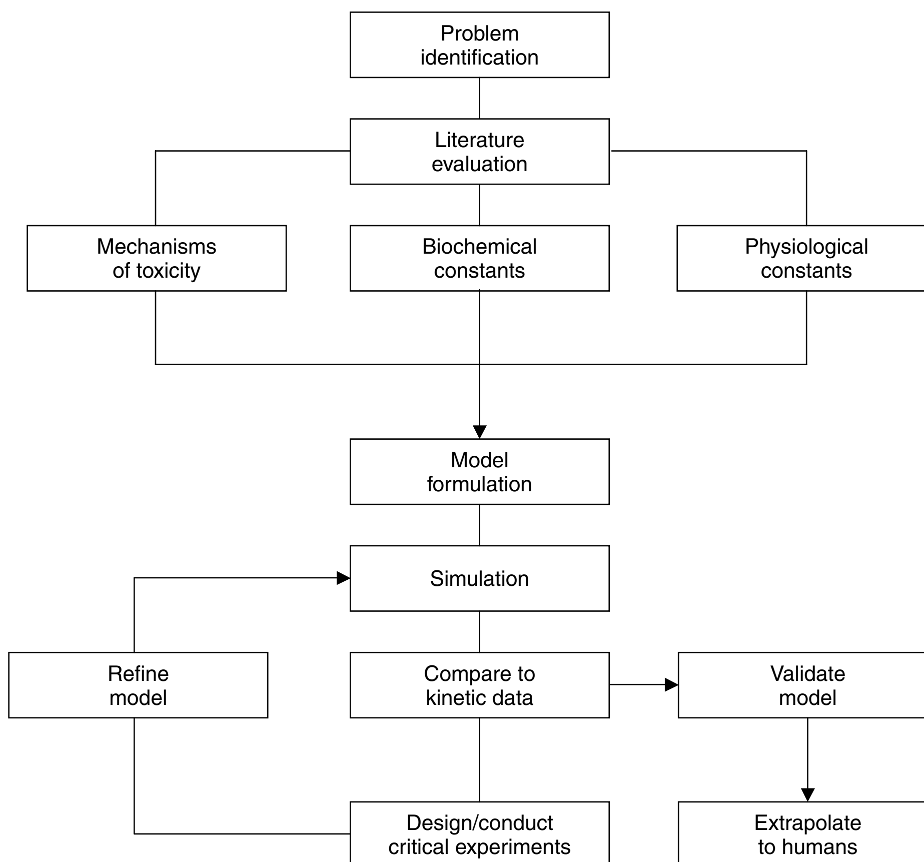

Figure 23.2. Flowchart illustrating processes involved in PBPK. (From M. E. Andersen, *Pharmacokinetics in Risk Assessment, Drinking Water and Health,* National Academy Press, Washington, D.C., 1987, pp. 8–23.)

of summary data such as means and standard deviations can be alternatives

- Care should be taken with modeling high-dose data (particularly extreme doses) because the model shape in the low-dose region can be influenced by high-dose data
- Log transformation of data should be justified because such a transformation may distort the dose-response curve
- Data variability should be described by appropriate statistical techniques and reflected in the potency estimate (e.g., by weighting the data in the fitting procedure)
- Confidence intervals or limits should be included in the analysis because they can be valuable for evaluating the influence of variability on the potency estimates
- An estimate for the uncertainty of the model used in the analysis should be included
- The statistical fitting method used must be clearly described.

For the most well-studied chemicals or drugs, it is likely that the biochemical constants such as K_m's and $V_{\max}$'s are known and readily retrievable from the information data base. However, it must be made clear here that the K_m, $V_{\max}$, and K_F (first-order rate constant) in a PBPK model (known as in vivo K_m, $V_{\max}$, K_F for a given chemical) such as the ones given in Figure 23.1 are hybrid constants of all the saturable or linear metabolic pathways, respectively, for the chemical of interest in the organ and/or body. They are different from the in vitro K_m, $V_{\max}$, or K_F of a given pure enzyme. While they are not directly interchangeable, the in vitro constants in the literature may be used to estimate in vivo constants for modeling purposes (Kedderis and Lipscomb, 2001; Lipscomb et al., 1998). Also, for most well-known chemicals, it is likely that enough is known about the mechanism of toxicity to be incorporated into the model for computer simulation. Physiologic constants such as organ volumes and blood flow rates are usually available in the literature for the common laboratory animals as well as humans. Therefore, at least in those instances of "well-known" chemicals, a

model may be conceptually illustrated as in Figure 23.1 and mathematically represented by a number of mass balance differential equations. Computer simulations may be made for any number of desired time course endpoints such as the blood levels of the parent compound, liver level of a reactive metabolite, and similar information on different species, at lower or higher dose levels, and/or via a different route of exposure. The experimental pharmacokinetic data may then be compared with PBPK model simulation to see if they are superimposable upon each other. If this is indeed the case, the model is consistent with actual results. Validation of the PBPK model with data sets other than the working set (or training set) to develop the model is necessary. Once validated, the PBPK model is ready for extrapolation to other animal species, including humans. However, if the experimental data and PBPK model simulation are not consistent, the model might be deficient because critical scientific information might be missing or certain assumptions are incorrect. The investigator, with knowledge of the chemical and a general understanding of the physiology and biochemistry of the animal species, can design and conduct critical experiments for refining the model to reach consistency with experimentation (Fig. 23.2). This refinement process may be repeated again and again when necessary; such an iterative process is critically important for the development of a PBPK model. There is always the possibility that a good model may not be obtained at the time because of the limitation of our knowledge on the chemical. An emphasis must be made here that the investigator and the knowledge possessed are the single most important determinant of the outcome of the results; mathematical modeling and advanced computational capabilities are nothing more than good tools.

MYTHS ABOUT PHYSIOLOGICALLY BASED PHARMACOKINETICS; ARE THEY REAL?

Common belief is that PBPK modeling is highly resource intensive and very difficult to do, particularly involving interactive pharmacokinetics or pharmacodynamics. This warrants some special discussion: First, by the time chemicals such as drugs or pesticides reach the stage of commercialism, many types of studies have already been conducted, including pharmacokinetic studies. Thus, they are already resource intensive during the developmental stage to become successful chemicals in commerce. The important point is how may quantitative, time course data useful for PBPK model development be generated during the product development phase? The data required for PBPK modeling are really not too much different from those required for the classical pharmacokinetics in the present IND/NDA (investigational new drug application/new drug application) process. A slight new orientation to the existing battery of studies would generate adequate quantitative time course data for PBPK modeling. Thus, it is definitely not any more resource intensive from the existing requirements. Furthermore, if the incentive (e.g., regulatory guideline-driven scientific studies) is there, such quantitative, time course data would have been automatically generated during the product development phase. In fact, PBPK modeling, being a hypothesis testing tool in toxicology, may be utilized to conduct many different kinds of experiments on a computer (i.e., in silico toxicology).

A great deal of research effort has been devoted to the development of *simple*, *high-throughput*, and in vitro predictive tools in the pharmaceutical industry (van de Waterbeemd and Gifford, 2003). While these short-term, rapid assays certainly offer some utility, particularly in the early drug development process, an inherent danger of such tools is the “by-pass” of integrated mammalian physiology and architecture of the body. In that regard, development of in silico toxicology such as PBPK/PD modeling and other biologically based computer modeling has the advantage of integrating whole-body pharmacokinetics and pharmacodynamics with computational technology. The resulting predictive tools will minimize unnecessary experiments and improve the *attrition rate* of drug or chemical product development with much more scientific validity and confidence. In that sense, PBPK/PD modeling, once integrated as a part of the product development plan, will actually save expenses and resources as well as minimize unnecessary animal experiments.

Second, while PBPK modeling is by no means a very easy technology, it is not any more difficult than some of the classical pharmacokinetic and statistical modeling carried out in the routine product development process. Furthermore, excellent training opportunities (e.g., www.cvmbs.colostate.edu/enhealth/cett/) are available, and the development of software is such that more and more user-friendly tools are going to be available.

PHYSIOLOGICALLY BASED PHARMACODYNAMIC MODELING

Using plain English, pharmacokinetics can be considered as “What the body does to the chemicals,” and pharmacodynamics can thus be considered as “What the chemicals do to the body.” Physiologically based pharmacodynamic (PBPD) modeling is therefore computer simulation of pharmacologic or toxicologic effects of chemicals or drugs based on the biology of chemical/drug–receptor interactions. From the point at which a chemical or a drug enters into the body to the point of pharmacologic or

toxicologic effect, it is a continuum of pharmacokinetics and pharmacodynamics. It is difficult to distinguish where pharmacokinetics end and pharmacodynamics start. When we consider the pharmacology or toxicology of a chemical or a drug, we must consider both pharmacokinetics and pharmacodynamics to have a full understanding. To consider either area alone is to understand only part of the picture.

PBPK modeling preceded PBPD modeling by many years because of the slower and later advances of mechanistic understanding of modes of action of chemicals and drugs. It is a natural course of evolution that PBPK blossomed and flourished first because it was developed based on the fundamentals of mammalian physiology, analytical chemistry, engineering principles of mass transport and mass balance, and desk-top computer hardware and software development. These were all well-developed areas in the earlier days. PBPD modeling is dependent upon the mechanistic basis of chemical/drug–receptor interactions, and the biology associated with it took time to mature. The recent trend is such that more and more PBPD modeling work is evident. PBPK modeling has matured to the point that research endeavors are centered around more complex systems (e.g., multichemical interactions, subcompartmentalization of organs) or special problem-driven models (e.g., dermal uptake of chemicals from showers, exposure dose reconstruction). As will be discussed later, the "delumping" of organ and tissue compartments and the linkage of PBPK models with other types of biologically based models may lead us to *second-generation PBPK/PD modeling*.

DATA REQUIREMENTS FOR PBPK OR PBPD MODELING

What are the specific data needed for building PBPK models? Obviously, well-conducted in vivo pharmacokinetic data are essential, and usually the more the data sets (e.g., different doses, routes, species), the better. In each study, time course blood and tissue concentration data are essential. These time course data should include at least the following tissues and organs: blood (or plasma if blood cell binding is not an issue), liver (organ of metabolism), kidney (representing well-perfused organs/tissues), muscle (representing slowly perfused organs/tissues), and target organ(s)/tissue(s). We also need other PBPK-modeling specific information, such as (1) physiologic constants, including body size, organ and tissue volumes, blood flow, and ventilation rates; (2) biochemical constants, including the chemical-specific metabolic rate constants such as $V_{\max}$ and K_m, partition coefficients for tissues; and (3) mechanistic factors such as target tissues, metabolic pathways, and receptor interactions. Enzyme kinetic data, particularly human data, of at least the key metabolic processes will be important for the PBPK model. In vitro determination of tissue partition coefficients and enzyme kinetic data is relatively straightforward and inexpensive. With modern genetic engineering technologies, many human enzymes are available commercially. Thus, heretofore unavailable human enzyme kinetic information for many of the environmentally important chemicals are within easy reach for many laboratories. These experiments should be performed.

For PBPD modeling, the data requirement is much more variable because of the many different types of chemical/drug–receptor interactions. Thus, it is much more of a compound-specific nature. However, as a rule of thumb, quality time course data on the key biologic processes to be modeled are essential.

PBPK OR PBPK/PD MODELS FOR CHEMICAL INTERACTIONS (INTERACTIVE PBPK OR PBPK/PD MODELS)

Human exposure to chemicals is rarely, if ever, to single chemicals. In the area of clinical pharmacology, adverse drug interactions are undoubtedly serious concerns. For instance, Lazarou et al. (1998) estimated that there were over 2.2 million cases of serious adverse drug reactions (ADRs) in hospital patients in 1994 in the United States and among these cases 106,000 were fatal. During their hospital stay, the patients in the survey statistics were given an average of eight drugs. Comparing with other statistics of causes of death, these investigators (Lazarou et al., 1998) indicated that ADRs became the fourth to the sixth leading cause of death for that year in the United States. Thus, it is important to discuss the issues related to the development of interactive PBPK/PD modeling (see particularly the example given later on the work of Kanamitsu et al., 2000).

From the perspective of interactive PBPK/PD modeling, two aspects need to be addressed: pharmacokinetic interactions and pharmacodynamic interactions. Because PBPD modeling is a relatively recent effort and few such models are available for even single chemicals, we will concentrate all our discussion on interactive PBPK models. The most ideal and scientifically defensible data requirement for establishing an interactive PBPK model is that each component chemical in the mixture already have its respective established PBPK model and that there are many pharmacokinetic data sets in laboratory animals as well as in humans available for each of these component chemicals. The interactive PBPK model is then built on the basis of known pharmacokinetic interactions. For instance, the component chemicals may inhibit each others' biotransformation. The individual PBPK models may

then be linked together at the liver compartment by introducing competitive inhibition (or other types of inhibition) terms in the mass balance differential equation.

In some cases, even more specific and stringent data requirements are needed; an example may be the ultralow-dose pharmacokinetic data in perinatal developmental stages of laboratory animals for extrapolation to human fetuses and babies using PBPK modeling. This area is still in its infancy and further development is anticipated.

APPLICATION OF PBPK/PD MODELING

Dosing Schedule of a Chemotherapeutic Agent

Methotrexate is a folate analogue and a well-known cancer chemotherapeutic agent. This is one of the chemotherapeutic agents which was studied extensively with PBPK modeling. The mechanism of toxicity is due to the binding of methotrexate to dihydrofolate reductase, a key enzyme in DNA synthesis, leading to cessation of DNA synthesis and cell death. In some organs (liver, kidney, intestine, and marrow) and many tumors, methotrexate undergoes metabolism to active polyglutamate derivatives (even more potent inhibitors for dihydrofolate reductase). Because these polyglutamates are retained in some tissues far longer than the parent compound, it has been suggested that this effect is of great importance for the antineoplastic property of methotrexate.

Table 23.1 shows the toxicity of methotrexate to mice under a variety of dosing schedules (Morrison et al., 1987). Obviously, toxicity does not directly correlate with total dose. Decreasing total dose by a factor of 117 (350/3) led to an increase, rather than a substantial decrease, in toxicity. In addition, the area under the plasma concentration–time curve (AUC), a frequently used pharmacokinetic parameter for bioavailability, did not correlate with toxicity. For instance, the AUC for a bolus dose of 350 mg/kg is about two orders of magnitude greater than that of the 96-h infusion at 0.8 μg/h•, yet toxicity is higher with infusion. • Q4

The reason for the above phenomenon turned out to be intimately associated with the pharmacokinetics of methotrexate (and its polyglutamate metabolites) and the threshold and length of time that dihydrofolate reductase is inhibited (thus inhibition of DNA synthesis). At a bolus dose of 350 mg/kg, even though there is a short period of very high blood and tissue concentration, the inhibition of DNA synthesis did not persist long enough to cause lethality in at least some of the mice. At an infusion rate of 0.8 μg/h• for 96 h, even though the blood and tissue levels were low, they were nevertheless high enough to cause sustained inhibition of DNA synthesis, which ultimately translated into higher lethality in the animals. These scientific discoveries eventually led to the revision of the methotrexate PBPK model by incorporating the inhibition of DNA synthesis into the model. • Q5

From the modeling perspective, the iterative process of making new scientific discoveries and then refining the PBPK model by incorporating such new information into the model is a wonderful illustration of what we discussed in relation to Figure 23.2. An even more significant illustration is the fact that a validated methotrexate PBPK model can be used to conduct all the experiments in Table 23.1 on a computer. With such complicated dosing schedules, it is apparent how the lives of hundreds of mice may be saved and how much time and resources may be diverted to other more efficient usages.

"Electronic Rats"

One of our earlier examples was the PBPK/PD modeling of a toxicologic interaction between kepone (also known as chlordecone) and carbon tetrachloride (CCl_4) based on mechanisms of interactive toxicity and the application of computer technology in acute toxicity studies. This was a collaboration among three research groups: Melvin E. Andersen, CIIT (presently CIIT Centers for Health Research); Harihara M. Mehendale, Northeast Louisiana

Table 23.1. Dosing Schedule Dependence of Methotrexate Toxicity in Mice

Dose (mg/kg)	Schedule	Total Dose (mg/kg)	Peak Plasma Concentration (M)	Effect
350	Single dose	350	10^{-3}	LD50
25	Twice daily	50	10^{-4}	LD50
3	Every 3 h, 5 times, rest 8 h, then every 3 h, 3 times	24	10^{-5}	>LD50[a]
0.5	Every 3 h, 20 times	10	10^{-6}	>LD50[a]
0.8 μg/h	Infusion 96 h	3	10^{-8}	>LD50[a]

[a] Higher toxicity than LD_{50}.

Source: Morrison et al., 1987.

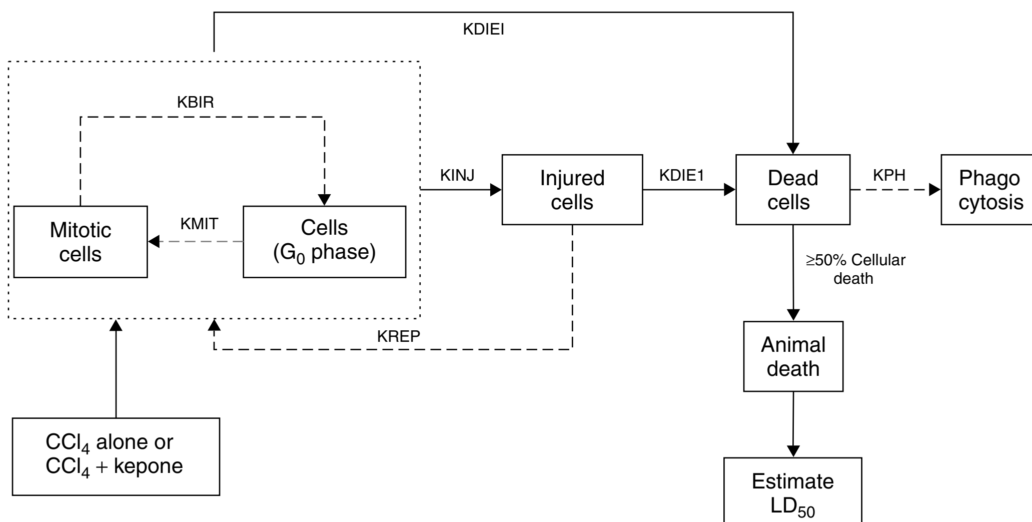

Figure 23.3. A conceptual PBPD model for CCl_4 and Kepone interaction. KMIT = rate constant for mitosis; KBIR = rate constant for cell birth; KINJ = rate constant for cell injury; KDIEI = rate constant for general cell death; KDIE1 = rate constant for cell death due to injury; KPH = rate constant for phagocytosis (El-masri et al., 1996).

University (presently University of Louisiana at Monroe); and our Quantitative and Computational Toxicology group at Colorado State University. The details of this study may be found elsewhere (El-masri et al., 1996). Briefly, CCl_4 is a well-known hepatotoxin. Following free-radical formation through the P450 enzyme system, the toxicity of CCl_4 can be an accumulation of lipids (steatosis, fatty liver) and degenerative processes leading to cell death (necrosis). Kepone is found in the environment as a result of photolytic oxidation of Mirex, a pesticide used for the control of fire ants, or as a pollutant from careless and irresponsible discharge. At relatively low levels (e.g., 10 ppm in the diet), even repeated dosing of kepone in the diet up to 15 days caused no apparent toxicity to the liver. The toxicologic interaction between kepone and CCl_4 was reported by Curtis et al. (1979). They illustrated that a 15-day dietary exposure of male rats to kepone at 10 ppm, an environmentally realistic level, markedly enhanced liver toxicity produced by an i.p. injection of a marginally toxic dose of CCl_4 (100 μL/kg). This toxicologic interaction is unique in that (1) unlike many other toxicologic interaction studies which were usually dealing with acute toxicity at very high doses, kepone in this instance was administered at a very low environmental level; (2) CCl_4 was also dosed at a low, marginally toxic level; and (3) the magnitude of toxicologic interaction, 67-fold, is very large. The mechanism of this toxicologic interaction was elucidated by Mehendale's group through a series of studies to be the impairment, by kepone, of the liver's regeneration process. These mechanistic studies were summarized in a number of publications (Mehendale, 1984, 1991, 1994).

As shown in Figure 23.3, a PBPD model was constructed by El-masri et al. (1996) based on the mechanism of toxicologic interaction between kepone and CCl_4. This PBPD model was verified by literature information, and it was capable of providing time course computer simulations of mitotic, injured, and pyknotic (dead) cells after treatment with CCl_4 alone or with kepone pretreatment. This PBPD model was further linked with Monte Carlo simulation to provide predictability of the acute lethality of CCl_4 alone and in combination with kepone. As shown in Table 23.2, the a priori predictions of lethality were in very good agreement with experimentally derived values except at very high CCl_4 levels. In this latter case, the underprediction of lethality was due to toxicity other than in the liver, that is, the neurotoxic effects of CCl_4 on the central nervous system. When this study was first presented at the International Congress of Toxicology Symposium in 1995, a reporter for *Food and Chemical News* wrote a section titled "Colorado Researchers Use Electronic Rats." Although it was somewhat amusing at the time, the term "*electronic rats*" nevertheless reflects our ultimate goal of in silico toxicology.

Clonal Growth in Relation to Carcinogenesis

The U.S. National Toxicology Program (NTP) and its predecessor, the National Cancer Institute's Carcinogenesis Bioassay Program, collectively, form the world's largest toxicology program (NTP, 2002). In its nearly 40 years of operation, fewer than 600 chemicals have been studied for carcinogenicity and other chronic toxicities

Table 23.2. PBPK/PD Modeling and Monte Carlo Simulation of Kepone/CCl_4 Toxicologic Interaction

• Q6

• Q7

Dose Observed[b] •		Model Prediction[a]			
Kepone (ppm)	CCl_4 (μL/kg)	Dead Rats	Percent Dead	Dead• Rats	% Dead
0	100	0	0.0	0	0.0
0	1000	1–2	13.2	1	11.1
0	3000	3	32.8	4	44.4
0	6000	4–5	47.8	8	88.8
10	10	0	0.0	0	0.0
10	50	4–5	47.5	4	44.4
10	100	8–9	84.0	8	88.8

[a] Mortalities in 48 h, given a hypothetical condition of $n = 9$; Monte Carlo simulation, $n = 1000$.
[b] Actual lethality studies ($n = 9$).
Source: Modified after Yang et al., *Toxicol. Lett.*, 82/82 : 497–504, 1995.

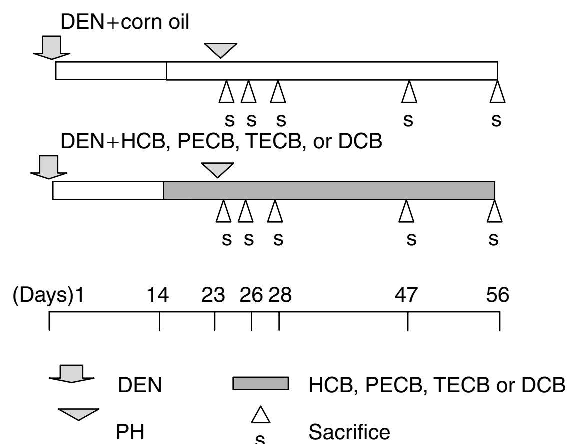

Figure 23.4. Experimental design for initiation–promotion study and estimated parameters for clonal growth model. The initiation agent, DEN, was administered i.p. (200 mg/kg) at week 0. HCB, PECB, TECB, or DCB was delivered by gavage starting week 2 at dose of 0.1 mmol/kg per day, 7 days/week. A two-thirds partial hepatectomy was performed on all animals on week 3. Liver tissues were collected 23, 26, 28, 47, and 56 days following initial DEN dosing. Concurrent controls without DEN initiation were also performed for all groups (Ou et al., 2003).

(NTP, 2002, 2003). These "gold standard" chronic toxicity/carcinogenicity studies are extremely expensive (i.e., up to several million dollars per chemical), require large number of animals (i.e., about 2000 animals per chemical), and are lengthy (i.e., 5–12 years per chemical). Thus, considering the approximately 80,000 chemicals in commerce (NTP, 2002), the number of compounds for which we have adequate toxicology information for risk assessment so far is miniscule. With the mode and rate of studying these chemicals as indicated above, it is unlikely that our society will ever have thorough toxicology information on the majority of the chemicals that we use now or may use in the future. Considering further the "real-world" issue of the health effects of chemical mixtures, it would be impossible to obtain adequate information on most of the chemicals or chemical mixtures that humans might be exposed to using the conventional approach (Yang, 1994, 1997). Thus, the PBPK/PD modeling approach described below represents a possible alternative to the expensive and time-consuming cancer bioassays.

We have used a modification of the medium-term bioassay of Ito and co-workers (1989a,b) to study the carcinogenicity of chlorobenzenes. The Ito assay involves the sequential administration of a potent initiator, diethylnitrosamine (DEN), followed by chemical treatment and mitogenic stimulation of hepatocyte growth via partial hepatectomy. As shown in Figure 23.4, this protocol allows the evaluation of carcinogenic potential within eight weeks by identification of glutathione *S*-transferase placental form (GST-P) positive preneoplastic foci as endpoint marker lesions. A large number of chemicals have been tested using this protocol. When compared with the two-year chronic bioassay, results from the Ito medium-term bioassay have correctly identified 97% of genotoxic hepatocarcinogens and 86% of the known nongenotoxic hepatococarcinogens (Ogiso et al., 1990). The principal modification of this protocol in our laboratory is the incorporation of pharmacokinetics and pharmacodynamics by conducting time course studies on tissue dosimetry, cell division rates, cell death rates, and GST-P foci formation.

To collect experimental data, briefly, as shown in Figure 23.4, male Fisher 344 rats, 8 weeks of age, were initiated with a single dose (200 mg/kg i.p.) of diethylnitrosamine. Two weeks later, animals were exposed to daily gavage consisting of 0.1 mmol/kg 1,4-dichlorobenzene (DCB), 1,2,4,5-tetrachlorobenzene (TECB), pentachlorobenzene (PECB), or hexachlorobenzene (HCB) in corn oil vehicle for 6 weeks. Partial hepatectomy was performed 3 weeks after initiation. Liver weight, 5-bromo-2′-deoxyuridine labeling index for analysis of cell division rate, and number and volume of GST-P positive foci were measured at 23, 26, 28, 47, or 56 days after initiation•. • Q8

We then used a clonal growth stochastic model (Ou et al., 2001, 2003; Thomas et al., 2000) to describe the dynamic growth of preneoplastic foci during the Ito medium-term bioassay. The clonal growth model is based

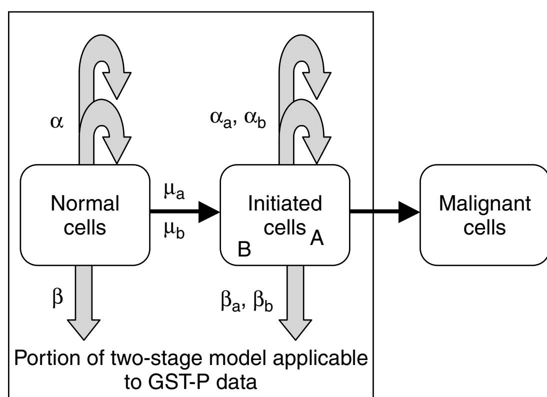

Figure 23.5. Simple two-stage model of carcinogenesis. The analysis presented here focuses on normal and initiated states (Ou et al., 2003).

on the two-stage model of carcinogenesis (Moolgavkar and Luebeck, 1990; Moolgavkar and Venzon, 2000), where the carcinogenesis process is described by the two critical rate-limiting steps: (a) from normal to initiated cells and (2) from initiated cells to malignant states (Fig. 23.5). The model allows the incorporation of relevant biologic information such as the kinetics of tissue growth and differentiation and mutation rates. The clonal growth stochastic model adopts a discrete-time numerical approach, where the time axis is decomposed into a series of time intervals, where parameters are allowed to change between but not with segments. To represent the multiplicity of the cellular states and the time-varying nature of the numerous cell behavior variables, the numerical model resorts to a recursive simulation. The growth of normal liver is described deterministically, whereas other cellular events use stochastic simulation. This approach facilitates description of the complex biologic process with time-dependent values.

A combination of experimental toxicology and computer simulation described above was used to analyze clonal growth of GST-P enzyme-altered foci during liver carcinogenesis in an initiation–promotion regimen for DCB, TECB, PECB, and HCB. The clonal growth stochastic model (Ou et al., 2001, 2003; Thomas et al., 2000) incorporating the hypothesis of two initiated cell populations (referred to as A and B cells) (Fig. 23.5) was successfully used to describe the foci development data for four chlorobenzenes (Fig. 23.6 presented as an example for HCB and PECB). The B cells are initiated cells that display selective growth advantage under conditions that inhibit the growth of initiated A cells and normal hepatocytes. A sensitivity analysis of model parameters indicated that the net growth rate of B cells during the two-week regenerative period following partial hepatectomy is among the most sensitive parameters for determining the final foci volume. Furthermore, the estimated values of this model parameter among four chlorobenzenes appear to be positively correlated with the induction of CYP2B1/2, CYP1A2, c-*fos*, enlarged liver, and final foci volume, indicating that examining effects of chemicals on regenerative responses following partial hepatectomy may be a way to understand the carcinogenicity potential of chlorobenzene compounds. While TECB, PECB, and HCB all increased significant foci volume, only HCB had effects on normal hepatocyte proliferation. The use of a two-cell hypothesis for the description of DEN control data (with partial hepatectomy) also indicated the presence of multiple phenotypes of initiated clones following DEN treatment, with resistant phenotypes arising during early carcinogenesis.

As initiation–promotion protocols are widely used in the study of carcinogenesis, the clonal simulation of foci growth, in combination with PBPK modeling, will be a useful quantitative tool for examining the time course of a dose–concentration relationship at critical target tissues and concentration-dependent pharmacodynamic changes at cellular levels during carcinogenesis.

HOW ARE PBPK/PD MODELS VALIDATED? HOW CAN THEY BE USEFUL PREDICTIVE TOOLS?

To develop a PBPK/PD model, quantitative time course experimental data sets are essential for comparisons with computer simulations. As indicated earlier, the goal and the hope are that computer simulations are superimposable with experimental data. The data sets used for model building are working sets (or training sets). For model validation, it is very important that data sets other than the working sets are used to compare with the model simulation results. For instance, assume• a PBPK/PD model was developed using PK and PD data sets from intravenous dosing of two dose levels of drug A reported by laboratory X. The validation of this PBPK/PD model for drug A should be carried out using different data sets such as PK and PD following oral and dermal dosing of drug A reported, respectively, by laboratories Y and Z. Generally, the more different data sets used in the validation process, the more robust the PBPK/PD model.

• Q9

Once a PBPK/PD model is validated, it may be used for predictive purposes. However, the present state-of-the-art is such that the predictive capability is limited to the very compound for which the PBPK/PD model is developed, or at the very least to close analogues with the same mechanism of action. For example, Kanamitsu et al. (2000), using a PBPK model, predicted in vivo drug–drug interaction between triazolam and erythromycin based on in vitro enzyme kinetic studies using human liver microsomes and recombinant human cytochrome P450 3A4

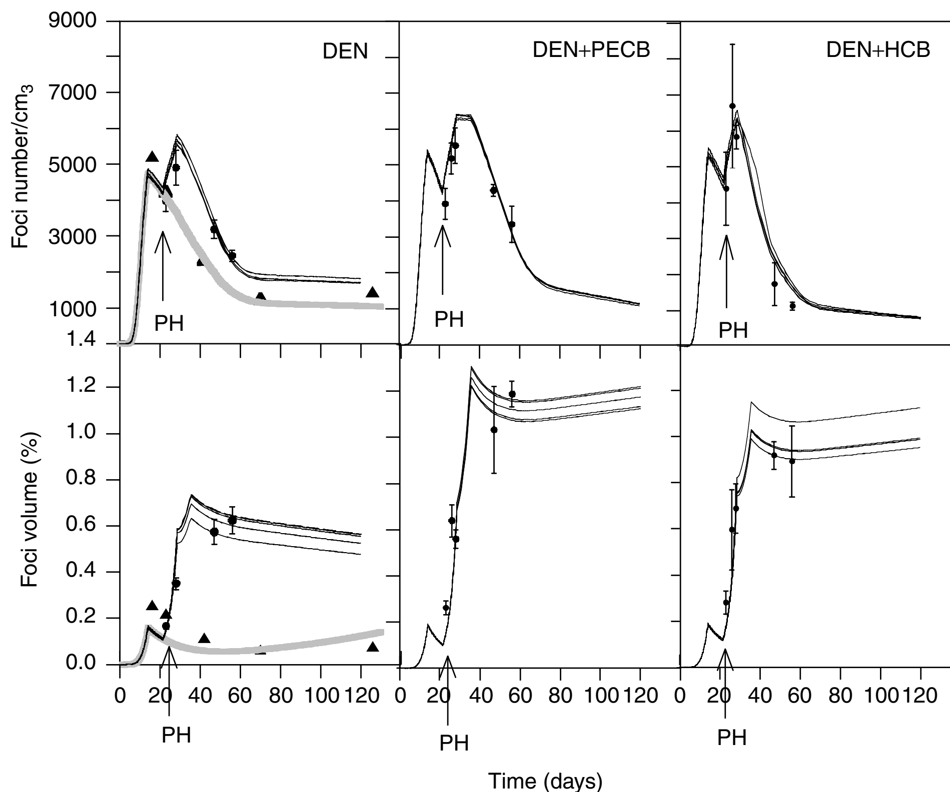

Figure 23.6. Comparison of the clonal model outputs with experimental measurements of foci growth for PECB and HCB congeners. Time-dependent changes in foci growth were measured in animals subjected to an initiation/promotion protocol using DEN as an initiator and PECB or HCB as a promoting agent. Using the standard stereologic methods, two-dimensional transection data of GST-P foci were converted to three-dimensional foci number (foci number/cm^3) and foci volume (volume percentage of foci) in the liver. To illustrate the stochastic nature of foci growth, the figures here show results from five runs of simulation. For comparison, experimental data of Jang et al. (1993)• (triangle symbols) and simulation results for the DEN controls without partial hepatectomy (lower solid gray lines) are shown along with those of the DEN controls (Ou et al., 2003).

• Q1

(CYP3A4). The mechanistic basis for this drug–drug interaction which involved 15 fatalities in Japan in 1993 is a *mechanism-based inhibition* (or "suicide inhibition," meaning the enzyme metabolizes a chemical to a reactive metabolite which, in turn, irreversibly inactivates the enzyme) for macrolide antibiotics, such as erythromycin. Once the enzyme is permanently inactivated, its metabolism of the other coadministered drug is impaired, leading to a scenario of "drug overdose." With the present state-of-the-art, Kanamitsu et al. (2000) should be able to use their PBPK model to predict potential serious dose-limiting toxicities for combination therapies involving macrolide antibiotics and coadministered drugs metabolized by CYP3A4 provided certain basic PK parameters are known for the drugs involved as well as in vitro enzyme kinetic information using human CYP3A4. When more and more such mechanistic information is available for classes of chemicals, it is likely that reaction rules may be established for certain molecular attributes, and reaction network modeling (see the following section on second-generation PBPK/PD modeling) may be linked with PBPK/PD modeling such that more generalized prediction of toxicities might become a reality.

FUTURE PERSPECTIVES: IN SILICO TOXICOLOGY AND SECOND-GENERATION PBPK/PD MODELING

In essence, in silico toxicology means integrating computer modeling with focused, mechanistic animal experimentation such that experiments which are impractical (e.g., too large, too expensive) or impossible (e.g., human experiments with carcinogens) to perform are conducted on a computer. We believe that utilization of computer

modeling is essential in the studies of toxicology of chemicals/drugs, chemical/drug mixtures, and their risk/safety assessment. The area of biology, in general, will be well served by the application of computer technology as an alternative research method to conserve resources and minimize the killing of laboratory animals. Looking into the future, we believe that the linkage of PBPK modeling and "reaction network modeling," described briefly in the following two subsections, has the potential of providing a computer simulation platform for complex biologic systems involving chemicals/drugs, chemical/drug mixtures, and/or multiple stressors. For a more detailed discussion, the readers are referred to three publications (Liao et al., 2002; Yang et al., 2003a,b) from our laboratory.

BUILDING A SECOND-GENERATION PBPK/PD MODEL

In classical pharmacokinetics and physiologically based pharmacokinetics, human or animal bodies were often described by a few compartments. By *second-generation PBPK/PD modeling*, we refer to integrating PBPK with reaction network modeling, thus including many more compartments (i.e., a delumping process). Our thoughts may best be explained in this way: If one draws a parallel between an oil refinery, where application of the reaction network modeling approach has been very successful, and a human body, the individual processing units in the oil refinery may be considered as equivalent to the vital organs of the human body. Even though the cell or organ may be much more complicated, the complex biochemical reaction networks in each organ may be modeled and linked much the same way as the modeling of the entire oil refinery through linkage of the individual processing units.

REACTION NETWORK MODELING

From the perspective of its original application in petroleum engineering, a *Reaction Network Model* is a tool for predicting the amounts of reactants, intermediates, and products as a function of time for a series of coupled chemical reactions (potentially numbering in the tens of thousands of reactions for some systems). It is usually a mathematical or symbolic formulation suitable for solution on the computer. A reaction network model builder is a tool for the computer generation of a reaction network model. The model builder can thus be used not only to solve the kinetic equations of interest but also to generate the reaction mechanisms, rate constants, and reaction equations themselves.

Essentially, the model builder works as follows:

1. The concentrations of the species to be reacted or metabolized are input to the model builder.
2. For each species in turn, the model builder performs a test against each of a set of "reaction rules" to determine whether or not the species is susceptible to a particular chemical reaction.
3. If it is not susceptible to any reactions, no further action is taken on this species.
4. If it is susceptible, a transformation of the species into one or more product species is performed based on the particular chemical reaction.
5. Each of these product species then undergoes the same susceptibility tests and a similar transformation sequence. This leads to a linking of all reactants with intermediates and, ultimately, with final products. This linking forms the structure of the chemical "reaction network."
6. After the reaction network is established, the rate constants for the reactions are retrieved or are computed.
7. The coupled differential equations governing the reaction kinetics for the network are then formulated by the model builder.
8. Finally, the kinetic equations, that is, the model equations, are solved numerically, leading to the concentrations of all species as a function of time.

More details on reaction network modeling, particularly the initial application to biomedical research, are available in a number of recent publications (Klein et al., 2002; Liao et al., 2002; Reisfeld and Yang, 2003; Yang et al., 2003a,b).

CONCLUSION

It is fitting to conclude with some recent testimonials from reputable scientists for the importance of the integration of different fields and the central role computer modeling will play in biology:

In an AAAS Plenary Lecture on February 13, 1998, Dr. Harold Varmus, then Director of the National Institutes of Health, emphasized, among others, two specific themes: "Discoveries in biology and medicine depend on progress in many fields of science" and "Methods that dramatically expand biological data also demand new modes of analysis and new ways to ask scientific questions." He said, "In short, biology is not only for biologists."• • Q10

Craig Venter of the human genome fame stated, "If we hope to understand biology, instead of looking at one little protein at a time, which is not how biology works, we will need to understand the integration of thousands of proteins in a dynamically changing

• Q11 environment. A computer will be the biologist's number one tool."• (Bulter, 1999).

Tyson and colleagues (2001) indicated, "Many prominent molecular biologists have pointed out the pressing need for theoretical and computational tools to show the spatial and temporal organization implicit in the way the macromolecules are 'wired together' to create a living cell."• • Q12

PBPK models have proven useful in uncovering determinants of disposition of carcinogens and other compounds in the body; PBPD cancer models have shown the role of mutation and cell proliferation in the time course of tumor development. Both of these types of models initially lacked considerable biologic detail due to limitations of our knowledge of fundamentals of cell and molecular biology. The revolution in genomic technologies in the past decade has revolutionized the database to support mechanistic modeling of chemical disposition and of biologic responses and these technologies now provide a basis for expansion of these models with increasing biologic detail. Reaction network models of cell constituents and genetic regulatory networks of cellular controls appear particularly attractive candidates for approaches to uncover the interactions and perturbations controlling neoplastic transformations and growth.

Another contemporary extension of PBPK/PD models is closely tied to current attempts to unravel the circuitry of living cells and to discover the manner in which these circuits lead to biologic function and health. Mathematical models of gene networks and their perturbation by disease or by chemical exposures are now being developed (Andersen and Barton, 1999). In some cases, simple prokaryotic cells with specific circuit elements (e.g., biologic oscillators, switches, amplifiers) have been produced and examined by laboratory experiments and by computation (Guet et al., 2002; Hasty et al., 2002; McMillen et al., 2002). These computational models evaluate the protein networks within cells, the genetic control of these networks, and the logic of cellular responses affected by these networks (Alm and Arkin, 2003; Davidson et al., 2002; Ferrell, 2002).

Gene network modeling in intact animals will inevitably draw on PBPK and reaction network models to track concentrations of endogenous and exogenous signaling compounds and on PBPD models to simulate consequences of the interactions of these compounds with signaling pathways within cells. Reverse engineering approaches attempt to uncover the circuitry of working cells (Csete and Doyle, 2002). Large-scale simulation modeling that has formed the major core of PBPK/PD models remains important in examining these genetic regulatory networks. However, these types of models are being augmented by Boolean approaches using on–off logic to increase the scope of genomic coverage (Bolouri and Davidson, 2002). Efforts in understanding genetic networks may be especially important in providing insights into neoplastic diseases in which cell signaling networks become impaired or deranged (Hahn and Weinberg, 2002; Hanahan and Weinberg, 2000).

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