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Abbreviations

ADI	acceptable daily intake
ADR	adverse drug reaction
AhR	aryl hydrocarbon receptor
AIDS	acquired immunodeficiency syndrome
ATSDR	Agency for Toxic Substances and Disease Registry
BMDL	lower bound benchmark dose
BRN	biochemical reaction network
CAG	cumulative assessment group
CDC	Centers for Disease Control and Prevention
CMG	common mechanism group
FQPA	Food Quality Protection Act
GST-P	placental form of glutathione *S*-transferase
i.p.	intraperitoneal
LOAEL	lowest-observed adverse effect level
MCMC	Markov Chain Monte Carlo
NCEA	National Center for Environmental Assessment
NERL	National Exposure Research Laboratory
NHEERL	National Health and Environmental Effects Research Laboratory
NIEHS	National Institute of Environmental Health Sciences
NOAEL	no-observed adverse effect level
NRMRL	National Risk Management Research Laboratory
NTP	National Toxicology Program
OCDD	1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin
OP	organophosphorus
OPP	Office of Pesticide Programs
PBPK/PD	physiologically based pharmacokinetic/pharmacodynamic
PCB	polychlorinated biphenyl

ppb	part per billion
QD705	quantum dot 705
QSAR	quantitative structure–activity relationship
RSM	response surface method
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TSCA	Toxic Substances Control Act
USEPA	U.S. Environmental Protection Agency
UV	ultraviolet
VOC	volatile organic chemical

1.09.1 Introduction

Former Secretary of Defense Donald Rumsfeld once said (Shermer 2005), regarding intelligence reports, "There are *known knowns*. There are things we know we know. We also know there are *known unknowns*. That is to say, we know there are some things we do not know. But there are also *unknown unknowns*, the ones we don't know we don't know." Rumsfeld's wisdom on intelligence appears to apply perfectly to the state of chemical mixture toxicology. Among the three categories, the *unknown unknowns* are the ones that we worry about the most in the area of chemical mixture toxicology.

In July 2005, Centers for Disease Control and Prevention (CDC) released its Third National Report on Human Exposure to Environmental Chemicals (CDC 2005). This Third Report, similar to its two predecessors but with expanded effort, contains exposure data for the U.S. population for 148 environmental chemicals over the period 2001–02. It also included the results from the 1999–2000 exposure data in the Second Report. The sample size in the Third Report, in general, ranges from a few hundreds to a few thousands, with a low of 210 samples for 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDD) analyses and a high of 8945 for cadmium or lead analyses. With such large sample sizes, as well as the obviously meticulous work, the published results undoubtedly represent the general U.S. population. Like the earlier reports, the CDC went out its way to emphasize that "the measurement of an environmental chemical in a person's blood or urine does not by itself mean that the chemical causes disease." This statement, cautious from the point of view of a governmental agency responsible for public health, does not offer much comfort in explaining what is the significance of the presence of one or more of such chemicals in our body. Given the fact that the 148 chemicals analyzed in the serum or urine samples were from the same individuals, an even more important question to ask is: What is the toxicological significance of the presence of such a 'cocktail' (mixture) of chemicals in our body? In many ways, this is the kind of *unknown unknowns* on chemical mixture toxicology that should worry those of us in the science of toxicology.

None of the scientists active in toxicology will pretend to know all the insights and answers. There are many *unknown unknowns* to us as well. However, with the collection of expertise and experience in science, we should certainly think analytically about this nagging question of "What is the toxicological significance of the presence of such a mixture of chemicals, albeit at very low concentrations, in our body?"

No one knows for certain, but we could look at this from two entirely different perspectives. One conclusion that we could make is that they (i.e., these chemicals in our body) are merely nuisance; it is a price that we pay for having a modern living in an industrialized society! They are necessary evils but they do not have any toxicological significance because they are present in our body at very low levels. Moreover, we do not witness any catastrophe and the average life span in our society is increasing. After all, there are scientists who believe that a small amount of any chemicals might have certain beneficial effects (Calabrese 2008; Calabrese and Baldwin 2003; Cook and Calabrese 2007; Smyth 1967).

However, we could also be much more cautious by saying that the presence of these chemicals in our body represents the toxicological *unknown unknowns* and we should try to err on the safety side and assume that they are potentially harmful to us. Would some of the persistent chemicals such as metals, dioxins, and polychlorinated biphenyls (PCBs) keep on accumulating in our body, thereby creating higher and higher tissue concentrations as we continue to be exposed in small doses? Considering the possibility of lipophilic organic pollutants being concentrated in our milk and passing on to our babies, it would certainly make anyone worry.

It is very difficult to reach a consensus among scientists between the two schools of thoughts above. However, because of the uncertainties *involved* perhaps a more prudent approach would be to adhere to the latter philosophy, namely, it is better safe than sorry. That being the case, we should consider the

cumulative risk assessment process. Indeed, there is a specific section on that concept in this chapter.

Also, in this new edition, a section on the potential toxicities of nanoparticles is included. Nanotechnology is no doubt one of the most important technological advances in the twenty-first century. It was estimated that within the next few years, the worldwide business involving nanoparticles will reach $1 trillion (Hardman 2006). Many of these particles are metal–organic mixtures and very little is known about their potential health effects on humans and other species. In terms of toxicological interactions, nothing is known about them and yet they are actively marketed and used in our society already.

What is the real meaning of 'toxicologic interaction' in light of the recent advances in toxicology? Lindenschmidt and Witschi (1990) defined toxicologic interaction as "the combination of two or more chemicals that results in a qualitatively or quantitatively altered biological response relative to that predicted from the action of a single chemical. The interaction of the chemicals may be simultaneous or sequential and the biological response may be increased or decreased." This definition, while adequate with respect to chemical toxicologic interactions with the body, should probably be modified today to reflect a broader scope. As shown below, toxicologic interactions may result from chemical–chemical interaction, chemical–biological agent interaction, chemical–physical agent interaction, and biological–biological interaction. Thus, the real meaning of toxicologic interaction may be defined by modifying the Lindenschmidt and Witschi (Lindenschmidt and Witschi 1990) definition slightly: *Toxicologic interaction is the combination of two or more chemicals, biological, and/or physical agents that results in a qualitatively or quantitatively altered biological response relative to that predicted from the action of a single chemical or agent. The interaction of the chemicals, biological, and/or physical agents may be simultaneous or sequential and the biological response may be increased or decreased.*

The most well-known example of chemical–chemical interaction leading directly to toxicologic interaction in the body may be the formation of nitrosamines from nitrites and amine at low pH in the stomach (Calabrese 1991b). Of course nitrosamines are one of the most potent classes of chemical carcinogens. Another interesting example illustrates the potential of direct chemical–chemical interactions in mitigating toxicity (Lindenschmidt and Witschi 1990). In the London fog disaster of 1952, many cattle at the Smithfield Show held at Earl's Court developed acute respiratory signs. Upon necropsy of the 12 more seriously intoxicated animals, evidence of acute lung damage was found. Pigs or sheep in the vicinity, however, were not affected. The differential toxicity was eventually elucidated to be the direct chemical–chemical interaction between ammonia and SO_2. Pigs and sheep, being in not well-cleaned pens and/or closer to the ground, were protected from SO_2 toxicity because of higher concentrations of ammonia fumes.

In the broadest sense, chemical–biological interactions include pharmacodynamics/toxicodynamics of any toxicants. Thus, receptor-mediated toxicity such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin–aryl hydrocarbon receptor (TCDD–AhR)-derived toxicities as well as multistage carcinogenesis from environmental chemicals are part of toxicologic interactions in this category. However, the examples given below illustrate the actual cases of chemical–biological agent interaction in the body leading to serious toxicities. In laboratory studies, the ingestion of nitrosamines enhanced the formation of squamous cell carcinoma in rats with chronic pneumonia. The possible mechanisms might involve alterations of local immune competence and pulmonary carcinogen metabolism and clearance (Corbett and Nettesheim 1973; Lindenschmidt and Witschi 1990; Nettesheim and William 1974). At a much broader level involving ecological parameters, Porter *et al.* (1984) evaluated the combined effects of five variables (food/water, an immunosuppressant, a plant growth regulator, a virus, and an environmental contaminant) on the growth and reproduction of laboratory mice and deer mice. Using a fractional factorial experimental design, they demonstrated interactive effects among the variables tested. For instance, malnourished mice were more sensitive to virus exposure and environmental pollutants. These authors concluded that "Interactions of certain 'harmless' chemicals at low levels may prove deleterious than higher doses of 'dangerous' toxicants acting alone . . ."

Two examples are given below for chemical–physical agent interactions. In the National Toxicology Program (NTP) studies on the possible toxicologic interaction of a 25-chemical mixture of groundwater contaminants and whole-body irradiation on hematopoiesis (Hong *et al.* 1991, 1992, 1993; Yang *et al.* 1989), exposure of the chemical mixture to $B6C3F_1$ mice further reduced bone marrow stem-cell proliferation resulting from radiation injury following repeated whole-body irradiation at 200 rads. Even 10 weeks after the cessation of chemical mixture exposure when all hematological parameters were normal, a residual effect of the chemical

mixture may still be demonstrated as lower bone marrow stem-cell counts following irradiation (Hong *et al.* 1991; Yang *et al.* 1989). Another example relates interaction between pesticidal activity and ultraviolet (UV) light. It is commonly known that UV light will degrade hazardous chemicals including pesticides. However, a study by McCabe and Nowak (1986) demonstrated that some pesticides act synergistically when combined with UV light.

The area of biological–biological interactions is not well defined. Any infectious disease clearly involves biological–biological interactions inside the body. There are not yet very clearly defined examples for biological agents interacting with each other first and then to cause severe toxicity within an organism, although such biological–biological interactions are theoretically probable. Perhaps, the deadly acquired immunodeficiency syndrome (AIDS) and Ebola virus 'jumping' from intermediate host to humans may be considered as examples of biological–biological interactions prior to infecting humans.

1.09.2 Unique Issues Related to Chemical Mixtures

1.09.2.1 Chemical Mixtures Are Ubiquitous

What is a chemical mixture? The answer is that almost everything around us is a chemical mixture: a breakfast of bacon, eggs, orange juice, toasts, and coffee; a lunch of tuna salad sandwich with coke; a gourmet dinner of veal, mushrooms, asparagus, and wine; the suits and dresses we wear; the cosmetics, toiletries, and medicines we use; etc. Even our own body is a chemical mixture. Considering all these 'background exposures' to chemicals, there is really no such thing as 'single chemical exposure' in our life.

In contrast to this reality, however, in 1994, it was estimated that about 95% of the toxicology studies conducted had been with single chemicals (Yang 1994). This represents a very uneven distribution of research resources. Single chemical toxicology studies are important in terms of obtaining fundamental mechanistic information. However, regarding real-life issues of risk assessment of chemical exposures to human health, their (single chemical studies) utility is really limited at best. The past and present regulatory practices of considering single chemicals in the risk assessment process, particularly in environmental exposures of complex chemical sources, are inadequate. This sentiment, while advocated repeatedly by this author (El-Masri *et al.* 1995; Yang 1994a,b; Yang and Rauckman 1987; Yang *et al.* 1989, 1995), has been in the scientific literature for a long time; a number of quotes are given below to provide a glimpse of such sentiment.

> . . .a careful reading of many of the proceedings from conferences, workshops, and reports of expert committees reveals a repetitious restatement of the obvious: for example, humans are not exposed to single agents; the environment provides exposure to a complex daily mixture of agents; health standards have long ignored the issue of multiple exposures; and this should be an area of high priority. . .
>
> (Calabrese 1991b)

> . . .In the ambient air, we breathe mixtures of pollutants; therefore, potential interactions between inhaled toxicants should be an area of concern for setting of ambient air quality standards by regulating agencies. . . .
>
> (Gelzleichter *et al.* 1992)

> . . .Although human contact with ambient air pollution usually involves simultaneous exposure to more than one chemical, . . . experimental studies have routinely examined effects resulting from single pollutant . . . public health standard have generally been set without regard for potential interactions between the materials being regulated. . . .
>
> (Schlesinger *et al.* 1992)

More recent events, however, have been more encouraging. Thus, it is gratifying to note that the U.S. Environmental Protection Agency (USEPA) has adopted an official policy of advancing cumulative risk assessment. A later section in this chapter provides details of their program and progress.

1.09.2.2 Chemical Mixtures Are Real-Life Issues

This particular feature is best reflected by an example. A citizen's petition to the EPA in 1984 (USEPA 1985) is summarized to illustrate the real-life nature of toxicology of chemical mixtures and the reality of how ill-prepared the toxicology community is. On 17 July 1984, EPA received a citizen's petition, under section 21 of Toxic Substances Control Act (TSCA), from Robert Ginsburg, Ph.D. (representing Citizens for a Better Environment) and Mary Ellen Montes (representing Irondalers Against the Chemical Threat). These citizens asked the EPA ". . .to determine the immediate and cumulative health effects of multiple toxic substances from multiple

sources in air, land, and water in the Southeast Chicago area;..." Among the requests by these petitioners were the following items:

1. The petitioners requested that the Administrator determine the name and nature of business of each person and business entity in the Southeast Chicago area whose business includes the manufacture, distribution in commerce, processing, use, or disposal of any one or more of the following 'Identified Substances' detected in the air, water, and land of the area: coke oven emissions, benzene, chromium, arsenic, cadmium, nickel, toluene, xylene, acetone, copper, and lead.
2. The petitioners requested that the Administrator compel the persons and business entities identified above to commence testing of the Identified Substances and such other chemical substances and mixtures, as soon as practicable, the testing of which shall include the following environmental and health effects:

   A. The cumulative effect, over an extended period of time, of each Identified Substance individually and in combination with every other Identified Substance (i.e., benzene alone, benzene with chromium; benzene with chromium and arsenic, etc.);
   B. The synergistic/antagonistic effect of each Identified Substance in combination with every other Identified Substance, occurring at one time;
   C. The effect of multimedia exposure to each Identified Substance individually and in combination with every other Identified Substance;
   D. The cumulative, synergistic/antagonistic, and multimedia effect, as set forth above, for each and every other chemical substance and mixture which may create an unreasonable risk of injury to the residents' health or their environment...

Clearly, there were some challenges posed by this petition! While the toxicology 'establishment' devoted as much as 95% resources, energy, and talents to the knowledge on health effects of single chemicals in a 1994 estimate (Yang 1994), at least two public groups, presumably laypersons, were asking realistic questions 10 years earlier to which we, as toxicologists, had no answers. Even today, we still do not have satisfactory answers to those questions and requests.

1.09.2.3 Chemical Mixture Exposures Are Dynamic

Imagine a hazardous waste site under a variety of weather conditions. The sunlight, rainfall, wind, temperature, acidity/alkalinity of the soil, etc., all have their respective effects on the chemicals in the disposal site. Chemical–chemical interaction may happen; one extreme case would be combustion (i.e., an extreme form of oxidation). In such a case, new chemicals may be synthesized via oxidation. Consider, another instance, an indoor situation. The cooking, second hand smoking, the off-gassing from furniture, carpet, clothing, the application of pesticides, and insect repellants all contribute to indoor air pollution (Yang 1994). All the above activities change with respect to time. Thus, environmental exposure to chemical mixtures is a dynamic phenomenon.

Figure 1 is a real-life example of human exposure to auto-exhaust in Los Angeles (U.S. Department of HEW 1970). The dynamic nature of air pollutant

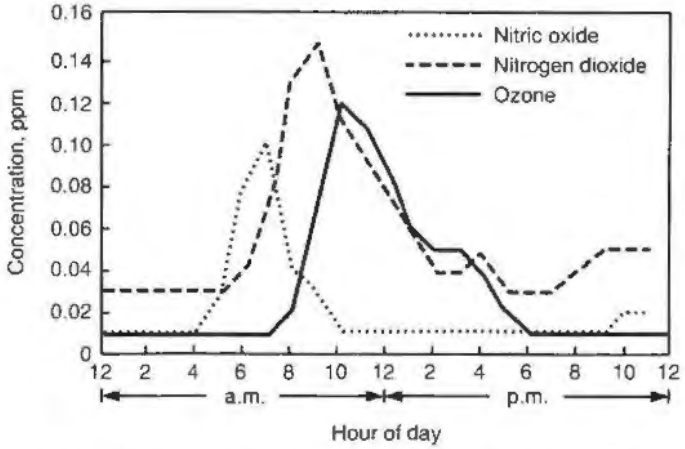

Figure 1 Diurnal variation of nitric oxide, nitrogen dioxide, and ozone concentrations in Los Angeles, 19 July 1965. Redrawn from U.S. Department of HEW 1970, Air Quality Criteria for Photochemical Oxidants.

levels because of diurnal variation of the traffic pattern is quite obvious.

1.09.2.4 The Immensity of Chemical Mixture Work

A major stumbling block toward advances in chemical mixture research is the immensity of the scope involved. For example, a chemical mixture with 25 component chemicals has $(2^{25} - 1)$ or 33 554 431 combinations (i.e., one chemical at a time, any two chemicals in combination, any three in combination, etc.) (El-Masri *et al.* 1995; Yang 1994). Even with this huge number of combinations, we have only considered one concentration per chemical or mixture. From a different perspective, there are about 80 000 chemicals (OTA 1995; Yang *et al.* 1998; Zeiger and Margolin 2000) being used in commerce. Just considering binary chemical mixtures, this means that there could be $80\,000 \times 79\,999/2 = 3\,199\,960\,000$ pairs of chemicals. If we consider all chemical mixtures for these 80 000 chemicals, the number of possible combinations becomes astronomical. Conventional toxicology methods for a systematic investigation of these chemical mixtures are beyond the reach of any laboratory/institution in the world.

From an entirely different perspective, as the number of chemical mixtures approaches infinity, the probability of toxicological interactions will approach unity. In other words, it becomes a certainty of the existence of toxicological interactions when the number of chemical mixtures approaches infinity much the same way as the possible presence of parallel universes as speculated by astronomers (Tegmark 2003). This concept might be appropriately termed as the 'Toxicology of Infinity.'

1.09.2.5 Toxicologic Interactions: Frequency, Concentration, and Threshold

In the above example of binary mixtures for the 80 000 chemicals in commerce, even if we assume that only one in a million of these pairs of chemicals act synergistically or have other toxicologic interactions, there would still be 3199 binary chemical mixtures possessing toxicologic interactions. Thus, even strictly on the basis of probability, we may expect a reasonably good chance to encounter toxicologic interactions in our daily life. However, the probability of encountering toxicologic interactions is far better than one in a million. The frequency of occurrence of toxicologic interactions may be further reflected by the fact that over 30 years ago more than 200 adverse drug interactions were known to occur as a result of the administration of two or more central nervous system depressant drugs (Zbinden 1976).

Multiple drug interactions in aging populations and in hospital patients are very serious concerns because of three reasons: (1) these segments of the society are more likely to be taking multiple drugs; (2) the dosing levels of the drugs are usually at higher concentrations, certainly not at low environmental pollutant levels; and (3) the increasing popularity of combination therapy or polypharmacy for given illnesses. The following two studies in the literature serve to illustrate the seriousness of toxicological interactions due to multiple drugs in our bodies.

Lazarou *et al.* (1998) reported in a meta-analysis that, in the year of 1994, over 2.2 million cases of serious adverse drug reactions (ADRs) occurred in hospital patients in the United States. During their hospital stay, the patients were given an average of eight drugs. Among these serious drug interaction cases, 106 000 were fatal, making ADRs the 4th to 6th leading cause of death for that year in the United States. In an experimental toxicology study, Jevtovic-Todorovic *et al.* (2003) administered to 7-day-old infant rats a combination of drugs commonly used in pediatric anesthesia (midazolam, nitrous oxide, and isoflurane) in doses sufficient to maintain a surgical plane of anesthesia for 6 h. They observed that such a common combination therapeutic practice caused, in their infant rats, widespread apoptotic neurodegeneration in the developing brain, deficits in hippocampal synaptic function, and persistent memory/learning impairments.

Looking at the angle of realistic exposure scenarios, other than occupational accidents, the concerns for health hazards from environmental contamination are related to low-level, long-term exposures. Most practicing toxicologists would probably consider that toxicologic interactions are unlikely at low environmental exposure concentrations. This is due to the common belief that these concentrations, usually at part per billion (ppb) levels, are far below the saturation levels for most biological processes, particularly the detoxifying enzyme systems. Are these common beliefs true? To answer this question, Yang (1994) went through some calculation for 1 ppb chloroform in drinking water due to chlorination disinfection process. He indicated that this level of chloroform means there are more than 5 quadrillion molecules in 1 l of water. Using a series of illustrations and arguments, Yang (1994) concluded that: (1) even

at 1 ppb level, there are a huge number of molecules in our body; (2) these molecules are not present 'alone' in the sense of chemical species, but they are present along with other xenobiotics; (3) there is a very narrow range of probably less than 3 orders of magnitude between 'no effects' and 'effects' in the various toxicity studies; (4) toxicologic interaction(s) seems possible, at least theoretically, at low exposure concentrations; however, the sensitivity of detection may pose a problem. His contention was, in part, supported by some findings particularly the clear dose-related *in vivo* cytogenetic toxicity in rats treated with an 'ultra low' concentration (i.e., ppb levels) of pesticide/fertilizer mixture (Kligerman *et al.* 1993). To offer some counter arguments though, it is instructive to refer again to the CDC human biomonitoring results of the presence of at least 148 chemicals, at low levels, in our bodies (CDC 2005). We have all these chemicals in our bodies and yet the general health of the population is good and the lifespan continues to improve. This is indeed one of the 'unknown unknowns' of toxicology.

Is there such thing as an 'Interaction Threshold'? Theoretically, there should be. In fact, El-Masri *et al.* (1996) studied the toxicologic interaction between trichloroethylene and 1,1-dichloroethylene using physiologically based pharmacokinetic/pharmacodynamic modeling and derived an Interaction Threshold of about 100 ppm based on pharmacokinetic changes. When two or more interactive chemicals are studied together, theoretically, there could be infinite interaction thresholds depending on the dose levels used for the individual chemicals in the studies (Yang and Dennison 2007). However, if we specify certain occupational or environmental exposure concentrations for all the other component chemicals in the mixture except one, we may obtain an interaction threshold for that set of specific exposure conditions (Yang and Dennison 2007). The interrelationship of 'thresholds' between chemical mixtures and their respective component single chemicals was studied by Yang and Dennison (2007) using three sets of data and two types of analyses. Their analyses revealed that the mixture 'Interaction Thresholds' appear to stay within the bounds of the 'Thresholds' of its respective component single chemicals. Although such a trend appears to be emerging, nevertheless, Yang and Dennison (2007) cautioned that their analyses were based on limited data sets. They urged that further analyses on more data sets, preferably the more comprehensive experimental data sets, are needed before a definitive conclusion can be drawn.

1.09.2.6 Chemical Mixture Research Offers Unique Opportunities

As mentioned earlier, according to a rough survey in the early 1990s (Yang 1994), most of the toxicology studies conducted to that time were carried out using single chemicals. One important reason for the lack of studies on chemical mixtures is the difficulty, complexity, and controversial nature involved. However, for the same reasons, there are also great opportunities in engaging this area of research because (1) it involves real-life issues and it is highly relevant to our society; (2) it is challenging, stimulating, and interesting (never boring!); (3) it is gratifying; and (4) there are few competitors in this area.

1.09.3 Methodological Advances for Assessing Toxicology of Chemical Mixtures

The NTP and its predecessor, the National Cancer Institute's Carcinogenesis Bioassay Program, collectively form probably the world's largest toxicology program (NTP 1989). In its over 46 years operation, under 600 chemicals have been studied for carcinogenicity and other chronic toxicities (NTP 2008). These chronic toxicity/carcinogenicity studies are extremely expensive (i.e., up to several million dollars per chemical) and they require large number of animals (i.e., about 2000 animals per chemical) and are lengthy (i.e., 5–12 years per chemical). Even though these studies are 'gold standards' of the world, considering the approximately 80 000 chemicals in the commerce (OTA 1995; Yang *et al.* 1998; Zeiger and Margolin 2000), the number of chemicals for which we have adequate toxicology information for risk assessment so far is minuscule. At the mode and rate of studying these chemicals as indicated above, it is doubtful 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 issue of health effects of chemical mixture exposure (i.e., real-world issues), it is impossible to adopt the approach of systematic conventional toxicology/carcinogenicity testing (Yang 1994).

From a different perspective, in recent years, concerns over animal rights have raised the

consciousness of many biomedical researchers regarding animal experimentation. According to the U.S. Office of Technology Assessment, some 17–22 million animals are used annually in the laboratories in the United States for research and testing (Klausner 1987). Another estimate put this number at about 20 million animals annually, 90% of which are rats and mice (Morrison 1993). However, animal rights groups estimate the toll to be even higher, as many as 70–100 million animals being sacrificed every year (Klausner 1987). Which number is the correct one is beside the point. The fact is that a staggering number of animals are killed for biomedical research each year.

It is apparent that new, alternative, less animal-intensive, shorter-term, and less expensive methods must be developed if we were to have a reasonable chance to deal with the hundreds of thousands of chemicals, as well as the near infinite number of chemical mixtures, in the environment. Recent advances indeed are heading toward that direction. Given below are some recent examples and the laboratories/institutions involved; these examples are selected based on one or more of the following criteria: (1) minimizing animal usage, (2) shortening experimental durations, (3) studying environmentally realistic concentrations, (4) utilizing statistical/mathematical modeling, (5) advancing efficient experimental designs, and (6) studying real-world problems.

To simplify the detection of carcinogenicity of chemicals and chemical mixtures, Ito and colleagues in Japan developed two types of medium-term (about 8–36 weeks) bioassays (Fukushima *et al.* 1991; Hagiwara *et al.* 1993; Ito *et al.* 1989; Roomi *et al.* 1985; Shibata *et al.* 1990; Tatematsu *et al.* 1985; Uwagawa *et al.* 1992). The first type is a Medium-Term Liver Foci Bioassay (Ito 1989), which utilizes the placental form of glutathione *S*-transferase (GST-P) as a marker for rat hepatic preneoplastic and neoplastic lesions (Roomi *et al.* 1985; Tatematsu *et al.* 1985). Ito's medium-term hepatocarcinogenesis bioassay utilizes F344 rats which are given a single dose of diethylnitrosamine to initiate carcinogenesis and, after a 2-week period, are given repeated exposure to a test compound. At week 3, rats are subjected to partial hepatectomy to maximize promotion (i.e., cell proliferation). All rats are sacrificed at 8 weeks for evaluation of development of preneoplastic hepatocellular nodules by staining for expression of GST-P (Ito *et al.* 1989). Extensive testing has demonstrated that the induction of GST-P positive foci in the medium-term bioassay for liver carcinogens correlates well with the incidence of hepatocellular carcinomas in parallel long-term assays (Ito *et al.* 1989). For rapid screening of large numbers of chemicals and for reduction of the use of large numbers of animals, this assay is of great advantage. The second type consists of a number of variations, but they are collectively designated by Ito and colleagues as the Medium-Term Multi-Organ Carcinogenesis Binassay or a Wide Spectrum Organ Carcinogenesis Model (Fukushima *et al.* 1991; Hagiwara *et al.* 1993; Shibata *et al.* 1990; Uwagawa *et al.* 1992). Although there have been a number of experimental protocols, in general, three to five initiators are given to F344 rats via various routes (e.g., intraperitoneal, subcutaneous injections, gavage, drinking water) in a 4-week period and followed by test chemical treatment or holding period of 12–20 weeks (Fukushima *et al.* 1991; Shibata *et al.* 1990; Uwagawa *et al.* 1992). In one protocol (Hagiwara *et al.* 1993), test chemical exposure was carried out first for 8 weeks. This was followed by 4-week treatment of three initiators. The subsequent holding period was for an additional 24 weeks. The endpoints in all of these models are histopathologic evaluation of preneoplastic and neoplastic incidences in multiple organs including nasal cavity, tongue, lung, esophagus, forestomach, glandular stomach, small intestine, large intestine, kidney, liver, thyroid, urinary bladder, and seminal vesicle. In all the above-mentioned assay systems, both the use of the animals and the experimental durations are reduced drastically.

The utility of the medium-term bioassay systems may be illustrated by a report from Ito *et al.* (1995). In this investigation, carcinogenic activities of pesticide mixtures, at very low levels, were examined with medium-term carcinogenesis bioassay protocols using F344 rats. With the 8-week liver foci model, combined dietary administration of 19 organophosphorus (OP) pesticides and one organochlorine pesticide, each at acceptable daily intake (ADI) levels, did not enhance rat liver foci formation. However, the same pesticide mixture at 100× ADI significantly increased the number and area of liver foci. With the multi-organ carcinogenesis model, a mixture of 40 high-volume pesticides or a mixture of 20 suspected carcinogenic pesticides, at the ADI level for each component, did not enhance carcinogenesis in any organ in a 28-week study following pretreatment of five initiators (Ito *et al.* 1995). The authors suggested that, based on their studies, the safety factor of 100 appeared to be adequate in the

quantitative hazard evaluation of pesticides. While the above findings are seemingly good news for public health, the direct application of results from such a drastic system as the multi-organ carcinogenesis model to the protection of the public should proceed with caution. The actual consequences of the application of five initiators are largely unknown. As was probably the case with Ito and colleagues (Ito *et al.* 1995), one may assume that each of these potent agents acted independently. However, there could be the possibility of antagonistic interaction from these five initiators toward the subsequent pesticidal carcinogenicity. In the absence of a thorough investigation and validation of this model, such a system should be considered as an interesting experimental model to be used for research purposes only for the time being.

Another interesting approach to study chemical mixtures at low levels was advanced by Feron *et al.* (1995). These investigators used a fractional factorial design and carried out a series of short-term toxicity studies in rats using chemicals with the same or different target organs and with similar or dissimilar mechanism of action. Their main objective is to test the hypothesis that, as a rule, exposure to mixtures of chemicals at nontoxic doses of the individual chemicals poses no health concern. For instance, one of the studies reported was a 4-week oral (food or drinking water) toxicity study of a combination of eight chemicals (KNO_2, stannous chloride, $Na_2S_2O_5$, metaldehyde, loperamide, mirex, lysinoalanine, and di-*n*-octyltin dichloride) in rats. The high-dose level was such that each of the eight chemicals was given at the lowest-observed adverse effect level (LOAEL). Thus, the rats were exposed to eight LOAELs in combination for these respective chemicals. The next lower dose was at the eight no-observed adverse effect levels (NOAELs) in combination for these eight chemicals. The lowest two doses are at the 1/3 and 1/10 NOAELs (i.e., at 1/3 and 1/10 of the second dose level). From the perspective of public health, this is a very innovative and relevant design in that the NOAEL or LOAEL is the starting point of quantitative risk assessment. These authors (Feron *et al.* 1995) concluded that chemical mixtures did not appear to be distinctly more hazardous than the individual chemicals, provided that the dose level of each chemical in the mixture did not exceed its own 'no-observed adverse effect level.'

In the late 1960s, in their classical study of using isobolographic analysis for the interaction of chloral hydrate and ethanol with respect to righting reflex loss in mice (Gessner and Cabana 1970), Gessner and Cabana painstakingly obtained the comprehensive data set through very large-scale animal experimentation involving between 2000 to 3000 mice. During those earlier days, the principal disadvantage of the isobolographic method was considered to be its extensive data demand (Calabrese 1991b). In a later study, however, Carter *et al.* (1988) revisited the experimental design by Gessner and Cabana by applying the then current advances in mathematical statistics. These investigators were able to successfully reaching the same conclusion of synergy between chloral hydrate and ethanol by using only 234 mice. Carter *et al.* were able to achieve this level of efficiency by taking advantage of the fact that response surface methods (RSMs) are useful in the estimation and analysis of isobolograms which are the contours of constant response of the underlying dose–response surface. The interaction between the two drugs in mice was evaluated using the RSM approach by fitting the logistic model to quantal data.

Between 1983 and 1990, the National Institute of Environmental Health Sciences (NIEHS)/NTP, under an interagency agreement with the Agency for Toxic Substances and Disease Registry (ATSDR), developed the 'Superfund Toxicology Program.' As part of this endeavor, a special initiative on toxicology of chemical mixtures of environmental concern, particularly groundwater contaminants derived from hazardous waste disposal and agricultural activities, was implemented. From this research effort, an approach was advanced where chemically defined mixtures, between binary and complex, of groundwater contaminants from hazardous waste disposal or agricultural activities were studied at environmentally realistic concentrations. One other criterion was that these chemical mixtures had to also have potential for life-time exposure in human populations (Yang 1992, 1994). A great deal of resources and personnel was devoted to the toxicology of a 25-chemical mixture of groundwater contaminants from hazardous waste disposal sites and two pesticide/fertilizer mixtures (Yang 1992, 1994) at low ppb levels following exposures of varying periods of time. The details revolving around the NIEHS/NTP chemical mixture toxicology program were reported in a number of earlier publications (Yang 1992, 1994; Yang and Rauckman 1987; Yang *et al.* 1989). Results obtained so far in that program revealed that health effects ranged from no abnormal responses to subtle immunosuppression, myelotoxicity, hepatotoxicity, and cytogenetic changes

(Chapin *et al.* 1989; Germolec *et al.* 1989; Hong *et al.* 1991, 1992, 1993; Kligerman *et al.* 1993; NTP 1993a,b; Yang 1994; Yang *et al.* 1989). Similar findings with respect to toxicologic interactions (i.e., immunosuppression, enzyme induction and inhibition, carcinogenesis) from low-level, long-term exposures or following administration to environmentally realistic chemical mixtures were also reported from other laboratories (Chaloupka *et al.* 1993; Chaturvedi 1993; Hasegawa *et al.* 1989; Silkworth *et al.* 1993).

One of the most ambitious and significant research endeavor on toxicology of chemical mixtures in recent years is the USEPA "4-Lab study on drinking water disinfection byproducts," an interlaboratory collaborative research program under the Office of Research and Development involving many scientists. Since the initial publication in 2002 of an overview of this research program (Simmons *et al.* 2002), a series of papers have been published (Claxton *et al.* 2008; Crosby *et al.* 2008; Miltner *et al.* 2008; Narotsky *et al.* 2008; Rice *et al.* 2008; Richardson *et al.* 2008; Simmons *et al.* 2008; Speth *et al.* 2008; Teuschler and Simmons 2003). Given below is a synopsis of this research program.

The primary goal for this program was to evaluate real-world complex mixtures of disinfection byproducts formed in bromide-containing water. The basic elements of this research program involved:

1. Selecting a surface source water and determining the levels of bromide and iodide, spiking if needed;
2. Splitting the source water into two streams for pilot plant treatment by two disinfection processes (chlorination and ozonation) such that health assessment of disinfection by-products from these two processes can be made;
3. Concentrating (reverse osmosis) and transporting finished drinking water;
4. Analyzing extensively known disinfection by-products, total organic carbon and halides;
5. Conducting a battery of *in vitro* and *in vivo* toxicity studies with a targeted focus on reproductive and developmental endpoints;
6. Analyzing and modeling data for dose–response assessment;
7. Assessing risk posed by these complex mixtures of drinking water disinfection by-products.

Many scientists and engineers of various backgrounds from the following four laboratories at USEPA participated in this research: the National Health and Environmental Effects Research Laboratory (NHEERL), the National Risk Management Research Laboratory (NRMRL), the National Exposure Research Laboratory (NERL), and the National Center for Environmental Assessment (NCEA).

Since 1992, an interdisciplinary team of researchers has attempted to integrate toxicology of chemical mixtures with physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling and biochemical reaction network (BRN) modeling. This is one of the handful of laboratories in the world using such an integrated computational toxicology approach toward chemical mixture studies. The next section is devoted to this area of activities.

1.09.4 PBPK/PD and BRN Modeling in Chemical Mixture Toxicology

In the earlier sections, we discuss a number of very critical issues that face the toxicology community today, and more globally, the society at large: (1) humans, as well as other organisms in the ecosystem, are exposed to chemical mixtures; (2) our knowledge on toxicologic interactions in chemical mixtures is inadequate; (3) there are immense number of chemical mixtures in the environment and the present testing/research attitude and capacity are inadequate; (4) the conventional toxicology methods are impractical, or even impossible, to deal with toxicologic interactions of chemical mixtures; (5) there is growing concern for the huge number of animal lives sacrificed annually for biomedical research; and (6) the single chemical mind-set in the past and present risk assessment arena is inappropriate although the cumulative risk assessment initiative at the USEPA is a positive development. Considering all these issues, it is obvious that some form of 'Predictive and Alternative Toxicology' must be developed to handle the complex issues of toxicology of chemical mixtures.

Is 'Predictive and Alternative Toxicology' an achievable goal for chemical mixtures? The current state-of-the-science would suggest that the answer is yes! Since the toxic effects produced by xenobiotics in the body are mediated by interactions between the chemicals (and their metabolites) and the biological molecules or structures (DHHS 1986), understanding pharmacokinetics and pharmacodynamics of xenobiotics is therefore essential in toxicology. With the advent of PBPK/PD and other types of

biologically based computer simulation technologies, correlation of tissue dosimetry (i.e., quantitative and temporal descriptions of xenobiotic concentrations at target tissues or organs) with specific toxicities becomes an attainable reality. By linking the interactive chemical components in a chemical mixture at the level of pharmacokinetic and/or pharmacodynamic modeling, it is possible to deal with the health effects, collectively, of the component chemicals in a variety of chemical mixtures of interest (El-Masri *et al.* 1995, 1997; Klein 2002; Krishnan *et al.* 1994; Liao 2002; Reisfeld 2007; Verhaar 1997; Yang 1994, 1995, 1996, 1998, 2004, 2005). Over the last 16 years, significant resources have been directed to the development of a quantitative and computational toxicology program on chemical mixtures with the ultimate goal of establishing 'Predictive and Alternative Toxicology' (Liao 2002; Verhaar 1997; Yang 2004, 2007). To deal with chemical mixture issues effectively, we must utilize and integrate: (1) computational technology, (2) PBPK/PD modeling, (3) model-directed, unconventional, focused, mechanistically based, short-term toxicology studies; (4) the latest advances in biology; and (5) the other biologically based mathematical/statistical modeling (El-Masri *et al.* 1995, 1997; Klein *et al.* 2002; Krishnan *et al.* 1994; Liao *et al.* 2002; Reisfeld *et al.* 2007; Verhaar *et al.* 1997; Yang 1996, 1998;Yang *et al.* 2004, 2005).

A number of approaches have been advanced for reaching the ultimate goal of predictive and alternative toxicology for chemical mixtures. The details of these approaches were discussed elsewhere (El-Masri *et al.* 1995, 1997; Klein *et al.* 2002; Krishnan *et al.* 1994; Liao *et al.* 2002; Reisfeld *et al.* 2007; Verhaar *et al.* 1997; Yang 1996, 1998;Yang *et al.* 2004, 2005); interested readers are urged to consult those papers.

For the development of a 'Bottom Up' approach, it is instructive to discuss the toxicologic interaction of a binary chemical mixture (Kepone and CCl_4) as an illustration. Based on the mechanisms of toxicity of this interaction, PBPK/PD modeling was used along with other statistical/mathematical modeling tools to predict acute toxicity. Kepone and CCl_4 interaction was selected because (1) this binary mixture has dramatic interactions at environmental levels for one of the components, Kepone (at 10 ppm) (Curtis *et al.* 1979); (2) the mechanistic basis for this interaction has been thoroughly and elegantly studied by Mehendale and coworkers (Mehendale 1984, 1991, 1994).

CCl_4 is a well-known hepatotoxin (Plaa 1991). Following free radical formation through 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) (Plaa 1991). Kepone (also known as chlordecone) 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 (Menzer 1991). 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 (Lockard *et al.* 1983).

The initial report on toxicologic interaction between Kepone and CCl_4 was published by Curtis *et al.* (1979). They demonstrated that a 15-day dietary exposure of male Sprague–Dawley rats to Kepone at 10 ppm, an environmentally realistic level of contamination, markedly enhanced liver toxicity produced by an intraperitoneal (i.p.) injection of a marginally toxic dose of CCl_4 ($100\,\mu l\,kg^{-1}$). The magnitude of this toxicologic interaction, based on enhancement of CCl_4 lethality, is about 67-fold. The mechanism of this toxicologic interaction was elucidated to be the obstruction of the liver's regeneration process through energy depletion (Mehendale 1984, 1991, 1994).

A PBPK/PD model was first developed for this toxicologic interaction (El-Masri *et al.* 1995, 1996). As shown in **Figure 2**, the pharmacokinetic portion of the PBPK/PD model was an adaptation of the PBPK model of Paustenbach *et al.* (1998). Following initial verification of this PBPK model, it was then linked to a PBPD model (**Figure 3**), which was based on the mechanism of toxicologic interaction between Kepone and CCl_4. By incorporating cell birth/death processes into the PBPK/PD model, time course computer simulations of mitotic, injured, and pyknotic cells after treatment with CCl_4 alone or in combination with Kepone were carried out (El-Masri *et al.* 1995, 1996). Verification of the PBPK/PD model was carried out by comparing simulation results with existing time course data in the literature (Lockard *et al.* 1983a,b); as shown in **Figure 4** (El-Masri *et al.* 1996).

To be of value to 'Predictive and Alternative Toxicology,' this PBPK/PD model was coupled with Monte Carlo simulation, a statistical sampling methodology to incorporate biological variabilities to PBPK/PD modeling, to predict the acute lethality of CCl_4 alone and in combination with Kepone. In doing so, we were able to conduct acute toxicity

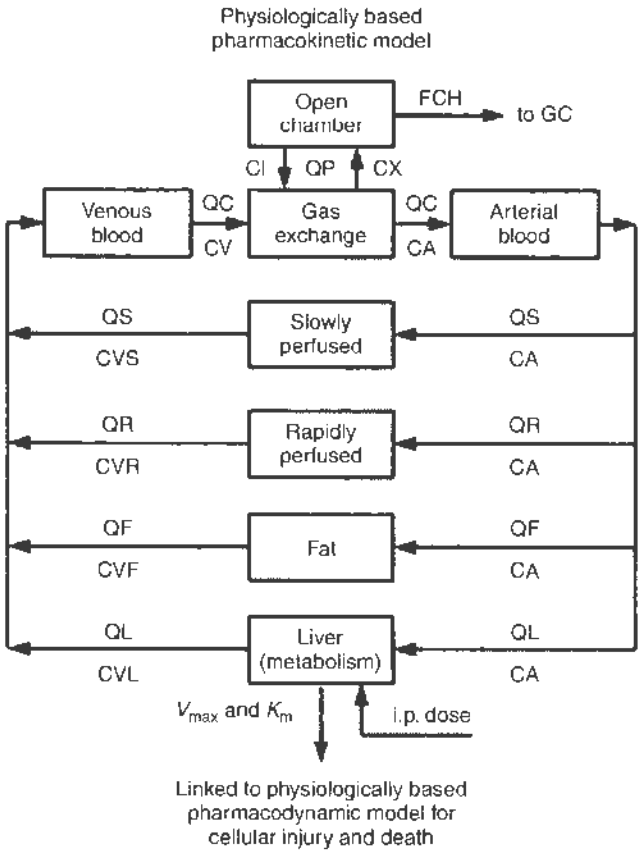

Figure 2 A PBPK model for CCl_4 adapted from Paustenbach, D. J.; Clewell, H. J.; Gargas, M. L.; Andersen, M. E. *Toxicol. Appl. Pharmacol.* **1988**, *96*, 191. CI and CX are concentrations of CCl_4 in the inhaled (thus chamber concentration) and exhaled breath. CV and CA represent venous and arterial blood concentrations of CCl_4. Q depicts blood flow rate. S, R, F, and L refer to slowly perfused, rapidly perfused, fat, and liver compartments, respectively. V_{max} and K_m are *in vivo* hybrid constants representing maximal velocity and affinity constants for enzyme systems involved in the metabolism of CCl_4. After El-Masri, H. A.; Thomas, R. S.; Benjamin, S. A.; Yang, R. S. H. *Toxicology* **1995**, *105*, 275.

studies on a computer with a very large sample (i.e., 1000 rats per dose) (El-Masri *et al.* 1996). The *a priori* predictions of lethality from PBPK/PD modeling or Monte Carlo simulation were in very good agreement with experimentally derived values except at very high CCl_4 dose levels (**Table 1**). In this latter case, the underprediction of lethality was due to toxicity in organs other than the liver. It is most likely a neurotoxic effect on central nervous system. Histomorphometric analyses of liver supported this explanation (El-Masri *et al.* 1996).

The above experiments and approaches represent the first step in the development of 'Predictive and Alternative Toxicology.' To recap the essentials, the above example illustrates that PBPK/PD modeling was used to correlate tissue dosimetry (i.e., in this instance, quantitative and temporal descriptions of CCl_4 concentrations at target tissues in liver) with hepatotoxicity leading to lethality. The coupling of Monte Carlo simulation incorporated biological variability such that the outcome, in this instance the predicted lethality, will be closer to reality. Even though this approach resulted in fairly accurate results, the PBPK/PD model for Kepone/CCl_4 interaction is by no means perfect. A thorough discussion on the possible refinement and improvement was beyond the scope of this chapter and the readers are referred to two other publications (El-Masri *et al.* 1995, 1996). However, a very important point to emphasize here is model-directed experimentation. PBPK/PD modeling will be at its most useful stage when it is utilized hand in hand with experimentation in an iterative manner. The real saving of animals and experiments may also come when model-directed experimentation is advanced to its fullest level.

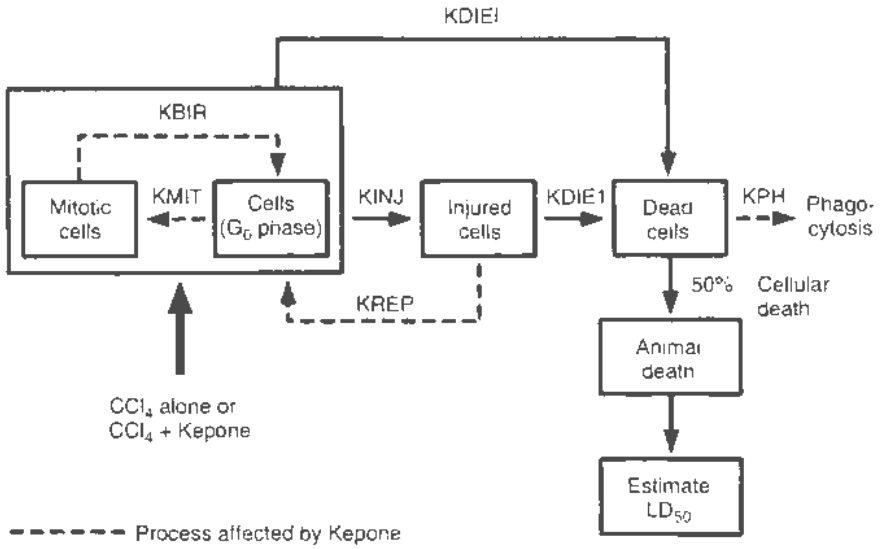

Figure 3 A PBPD model for toxicologic interactions between Kepone and CCl_4. This depicts the schematic of pharmacodynamic effects of CCl_4 on the cellular injury and death. The *dashed lines* depict the processes that are affected by the presence of Kepone. When cells are exposed to the reactive metabolites of CCl_4, their inherent death rate is influenced by two mechanisms. A major mechanism of cellular injury leading to death is through lipid accumulation which is illustrated here as the formation of injured cells and dead cells via two rate constants KINJ and KDIE1. For simplicity, all other causes of cell death including natural cell death and other CCl_4-related toxicities are lumped together into a hybrid constant KDIEI as a second mechanism. The injured cells can either be repaired (KREP) back to viable cells or continue to die. All dead cells, whether induced to die or injured to death, are removed from the liver by phagocytosis (KPH). Additionally, the PBPD model considers the effects of CCl_4, alone or in combination with Kepone, on cellular mitotic and birth rates (KMIT and KBIR). After El-Masri, H. A.; Thomas, R. S.; Benjamin, S. A.; Yang, R. S. H. *Toxicology* **1995**, *105*, 275.

One of the more important recent advances in the area of PBPK modeling is the Bayesian population PBPK modeling using Markov Chain Monte Carlo (MCMC) simulation. This is currently one of the most active scientific activities in PBPK modeling, particularly with respect to risk assessment. Pioneering efforts on Bayesian population approach to PBPK modeling are from F. Bois and colleagues (Bernillon and Bois 2000; Bois *et al.* 1996a,b) and F. Jonsson and colleagues (Jonsson 2001, Jonsson and Johanson 2001a,b, 2003). A dissertation by F. Jonsson (2001) at Uppsala University in Sweden provides a very nice discussion on PBPK modeling in risk assessment and the development of Bayesian population methods. The Bayesian population approach may best be explained by a passage from a 2003 publication by Jonsson and Johanson (2003):

> ...In a Bayesian analysis, the inclusion of previous knowledge is a fundamental and integrated part of the modeling process. The knowledge of model parameters before taking the present experimental data into account is quantified by assigning probability distributions, so called 'priors' to the parameters. These distributions are subsequently updated with regards to the data at hand. The resulting, so-called 'posterior probability distributions', or 'posteriors' for short, are consistent with both the experimental data and the priors, as the posteriors are derived as the product of the likelihood of the data and the prior probability of the parameters...

Until the early 2000s, Bayesian analyses were hampered by limitation of available methodologies. However, the availability of MCSim, a software in the public domain, and the advent of MCMC simulation greatly contributed to the recent surge of Bayesian analyses in PBPK modeling (Bois 2001, Bois *et al.* 2002). Thus far, Bayesian population PBPK modeling has been principally applied to single chemicals. However, as awareness increases on multiple chemical exposure being the rule rather than the exception and more and more experimental data are available on chemical mixtures, it is just the matter of time before Bayesian population PBPK modeling of chemical mixtures becomes the focus of research activities.

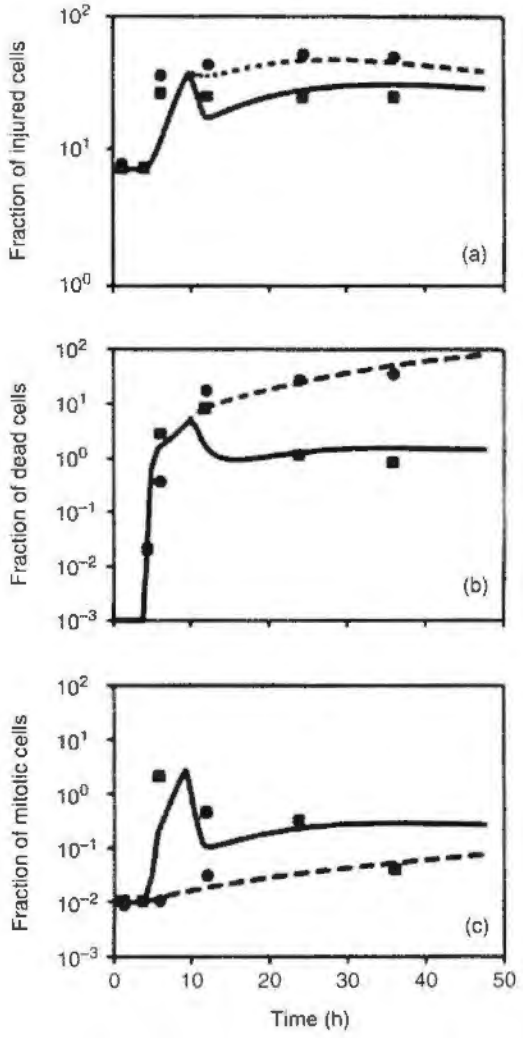

Figure 4 The PBPK/PD model predictions of (a) the injured, (b) pyknotic, and (c) mitotic cells from rats exposed to CCl_4 only (*squares* and *solid lines*) or CCl_4 with Kepone pretreatment (*circles* and *dashed lines*). The experimental data were obtained from Lockard, V. G.; Mehendale, H. M.; O'Neal, R. M. *Exp. Mol. Pathol.* **1983**, *39*, 246. The model predictions are given by the *lines*. After El-Masri, H. A.; Thomas, R. S.; Sabados, G. R.; Phillips, J. K.; Constan, A. A.; Benjamin, S. A.; Andersen, M. E.; Mehendale, H. M.; Yang, R. S. H. *Arch. Toxicol.* **1996**, *70*, 704.

The application of reaction network (RN) modeling technology to biological processes is a useful approach to these issues. Integrated with PBPK modeling, the BRN modeling is an approach that would hold the key in solving the problems of assessing chemical mixture toxicity. What is BRN modeling? How does it work? How is it integrated with PBPK modeling? And, how does it help to 'solve' the problems of assessing chemical mixture toxicity? Although more detailed answers to these questions are given elsewhere (Klein *et al.* 2002; Liao *et al.* 2002; Mayeno *et al.* 2005; Reisfeld and Yang 2007; Reisfeld *et al.* 2004; Yang 2004, 2005, 2007), a brief discussion of these questions follows.

BRN modeling has its origin in chemical and petroleum engineering. It was successfully employed in computer modeling and simulation of the complicated processes in oil refineries. In chemical or petroleum engineering field, an RN model is a tool that is used to predict 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). The RN itself is the interconnected, time-dependent series of reactions that occur in the system. In dealing with toxicology of chemical mixtures, we transplanted the concepts and technology of RN modeling to examine BRNs associated with the toxicological processes in an organism upon exposure to toxicants. Focusing on the role of BRNs in relation to the molecular events leading to toxicological changes in the body, the fundamental biological processes involved are as follows. First, mRNA, through the process of transcription, is derived from DNA (genomics). From mRNA, through the process of translation, proteins are formed (proteomics). Enzymes are functional proteins that catalyze reactions, creating BRNs (i.e., different pathways). The toxicants, once in the body, can affect any of the steps described above. Furthermore, these toxicants will undergo metabolic transformations themselves by the enzymatic pathways existed in the body, and some of their metabolites, being reactive species, will become new toxicants. The outcome of the dynamic balance of all these BRNs (metabonomics for intrinsic chemicals and xenobiotic metabolomics for extrinsic toxicants) determines the cellular physiology and toxicology. The term, biochemical reaction network (BRN) modeling, was principally derived based on the above description of the biological events.

How does the BRN modeling work? How is it integrated with PBPK modeling? And, how would it 'solve' the problems of assessing or predicting chemical mixture toxicity? The essential idea is that the BRN model software takes, as input, specifications for the reactants (usually in terms of their chemical structures), as well as the

Table 1 Kepone/CCl_4 mortality prediction by PBPK/PD modeling coupled with Monte Carlo simulation vs experimentally observed results

Dose given[a]		*Model predictions*		*Observed*[b]	
Kepone (ppm)	*CCl_4 ($\mu l\,kg^{-1}$)*	*Dead rats*	*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	9	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, $n = 9$; Monte Carlo simulation, $n = 1000$.
[b] Actual lethality studies ($n = 9$).
Source: El-Masri, H. A.; Thomas, R. S.; Benjamin, S. A.; Yang, R. S. H. *Toxicology* **1995**, *105*, 275.

enzymes (or other catalysts) involved. Inherent in the 'virtual enzymes' used in the modeling software are certain reaction rules, stipulating the nature of the relevant chemical and biochemical reactions. Algorithms within the software develop the associations between chemical species and create and solve the controlling kinetic equations in the reaction model. Thus, the output from the simulation is the detailed metabolic pathways (BRNs) showing the interconnections between the metabolites and the concentrations of all of these chemical species over time. As more and more information (e.g., chemical properties, chemical reaction mechanisms) is entered into the databases of the BRN model software, the predictive power of the software increases. At some point, the BRN model will 'grow' to the stage that it will be able to predict accurately the BRNs of a chemical mixture, be it a simple or complex one. An investigator, or a team of interdisciplinary scientists, can examine the nature and lifetimes of species of interest and, in the context of health risks, easily locate highly reactive species. Moreover, due to its design and flexibility, information can be fed back and forth between the BRN model software and the lower level (e.g., molecular level such as gene and protein expression) and higher level (organ/organism level) modeling tools such as gene network modeling or PBPK modeling to give a more complete picture of the risk.

The potential usage of BRN modeling as it is integrated with PBPK modeling will be discussed under Section 1.09.6.

1.09.5 Biochemical Mechanisms Underlying Chemical Interactions and Modulation of Response due to Chemical Interactions

The fundamental biochemical mechanisms involved in toxicologic interactions have been discussed by others (Calabrese 1991a,b; Goldstein *et al.* 1990; Kenakin 1993; Oesch *et al.* 1994) and a volume of *Environmental Health Perspectives* (1994) is almost entirely devoted to this subject. Therefore, the discussion here will be minimal. In general, the bases for interactions may be pharmacokinetics or pharmacodynamics. Pharmacokinetically based interactions include various stages in absorption, distribution, metabolism, and excretion. Pharmacodynamically based interactions include those between chemicals and receptor sites and critical cellular targets. In considering mechanisms of interaction, we should think much more broadly to include not only chemical–chemical interaction, but also chemical–biological, chemical–physical agent, and biological–biological interactions as discussed earlier in the Introduction.

Many factors can modulate the responses of toxicologic interaction; factors related to the agents, the exposure situation, the subject exposed, and the environmental conditions (Plaa and Vezina 1990). A number of examples are given below to illustrate these modulating factors.

The interaction between an agent and its receptor may be influenced by the presence of another agent. For instance, because of the similarity of molecular shape of coplanar PCBs and 2,3,7,8-TCDD, both will

bind with AhR. Even though the relative toxicities of coplanar PCBs are about 0.001–0.1 of that of 2,3,7,8-TCDD (Dewailly *et al.* 1991; Pollak 1993), the coplanar PCBs are present in much greater quantities, sometimes up to 10 000-fold higher, in biota than 2,3,7,8-TCDD and therefore they do represent a problem (Pollak 1993). The significance of the above information may be underscored by the findings that 2,3,4,2′,3′,4′-hexachlorobiphenyl, a coplanar PCB, enhanced cleft palate formation in mice by 2,3,7,8-TCDD (Birnbaum *et al.* 1985; Pollak 1993). In this instance, the PCB congener by itself did not cause cleft palate and 2,3,7,8-TCDD alone was significantly less potent.

The exposure situation and related complication may influence toxicologic interaction. Fluroxene was used in clinical medicine as an anesthetic agent safely for almost 20 years before the first fatal incidence (Kaminsky 1990). In 1972, a surgical patient who was an epileptic on a regimen of phenobarbital and diphenylhydantoin died within 36 h of operation due to massive hepatic necrosis (Kaminsky 1990; Reynolds *et al.* 1972). Based on experimental animal toxicology studies, the cause of death of this patient was attributed to potentiation of hepatotoxicity of fluroxene by phenobarbital and diphenylhydantoin through enzyme induction.

The intrinsic functions of the subject exposed to chemicals may modulate toxicologic interaction. Renal dysfunction may change drug disposition such that the likelihood of drug–drug interactions would increase. An actual clinical example is the interaction between aminoglycoside antibiotics and penicillins in patients with impaired renal function (Brater 1990). These antibiotics bind in solution to inactivate each other but the reaction is slow. Since penicillins are usually given in great molar excess to the aminoglycosides, the major consequence of such drug–drug interaction is inactivation of aminoglycoside to subtherapeutic concentration. This interaction, however, seems to occur only in patients with renal dysfunction. The reason was attributed to the retention of both the antibiotics in these patients with impaired renal function, thereby allowing sufficient time for this interaction to take place.

The best example for modulation of toxicologic interaction by environmental conditions may be the study by Porter *et al.* (1984) mentioned earlier in the Introduction. The finding that malnourished mice were more sensitive to virus exposure and environmental pollutants led the authors to speculate that, in the wild, food shortage for wildlife might cause added stress to weaken the animals' natural defenses against microbial infections and environmental pollutants.

Factors which modulate toxicologic interactions may be used to our advantage in preventive applications. In his research on hepatotoxicity from CCl_4 and other free radical-generating chemicals, Castro (1990) discussed the idea that prevention of hepatic necrosis can be achieved by the following possibilities: (1) through inhibition of metabolic activation to reactive metabolites; (2) through chemical trapping of necrogenic reactive metabolites; (3) through increased intensity of inactivating biotransformations; (4) through inhibition of lipid peroxidation; (5) through modulation of late stages of necrogenic process by manipulating protein synthesis and/or inhibition of degradative processes for proteins and phospholipids.

1.09.6 Risk Assessment Issues for Chemical Mixtures

The application of PBPK/PD to risk assessment of chemical mixtures may have several advantages: (1) the incorporation of mechanistic information on toxicologic interactions; (2) the conservation of resources and reduction of animal killing and suffering in the hazard identification step; and (3) the minimization of the necessity of using large uncertainty factors. Thus, PBPK/PD modeling will provide more realism into the risk assessment process. Of course, one must be aware of the fact that PBPK/PD modeling has its own intrinsic 'uncertainties'; therefore, as much as practicable, any PBPK/PD model must be rigorously validated with experimental results before 'Predictive Toxicology' so derived becomes meaningful. Given the recent advances and application of Bayesian statistics and MCMC in population PBPK modeling, there appeared to be a way to reduce such uncertainties.

The linkage of PBPK/PD and statistical/mathematical modeling with experimental toxicology of chemical mixtures will have great potential in application to risk assessment of chemical mixtures. A strategy for 'Predictive and Alternative Toxicology' for chemical mixtures and the development of 'Innovative Risk Assessment Methodologies for Chemical Mixtures' is shown in **Figure 5** (Yang 1997).

The basic concept is that using PBPK/PD modeling, toxicologic interactions in a chemical mixture

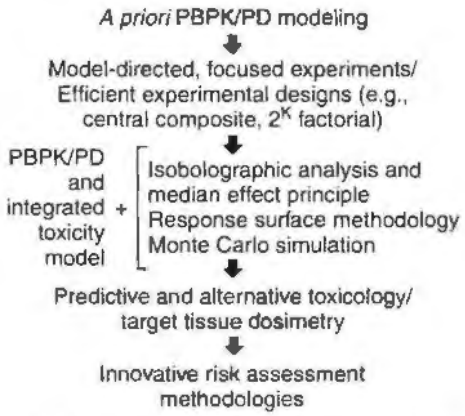

Figure 5 Our proposed strategy/approach to develop 'Predictive and Alternative Toxicology' and formulate 'Innovative Risk Assessment Methodology' for chemical mixtures. After El-Masri, H. A.; Thomas, R. S.; Benjamin, S. A.; Yang, R. S. H. *Toxicology* **1995**, *105*, 275.

may be linked at pharmacokinetic and/or pharmacodynamic levels. If necessary, repeated iteration of PBPK/PD modeling and model-directed experimental toxicology work may further improve and refine the PBPK/PD model for the chemical mixture. Efficient experimental design (e.g., central composite or 2^K factorial) may be utilized in this process to minimize the number of required experiments. Isobolographic analysis and/or response surface methodology will be used for the analysis of toxicologic interactions. With the aid of a technique such as Monte Carlo simulation, we may better predict tissue dosimetry at the pharmacokinetic and pharmacodynamic levels. Using such values as benchmark doses, human risk assessment of chemical mixtures may be carried out with less uncertainty.

While all these concepts discussed above are still valid, incorporating BRN modeling has served to update this approach. So far, PBPK modeling has handled up to five to six chemicals or lumped chemical components (Yang and Andersen 2005). To deal with much more complex mixtures, particularly on interwoven reaction pathways for individual chemicals in the mixture, the integration of PBPK modeling with BRN modeling is a promising tool (Mayeno *et al.* 2005; Reisfeld *et al.* 2007; Yang and Lu 2007; Yang *et al.* 2005, 2010). These integrated models would provide predictions of the fate of a chemical or chemical mixtures from the level of the whole organism down to molecular interactions (i.e., multiscale modeling) (Mayeno *et al.* 2005; Reisfeld *et al.* 2007; Yang *et al.* 2010). BRN modeling is currently at the research and development stage. When completed, chemicals or chemical mixtures with little or no animal toxicity data can be fed into the computer simulation program and their potential adverse health effects deduced from the metabolic RNs generated.

Despite the seemingly impossible complexity involved, an approach was proposed recently for predicting toxicities for any chemical mixtures (Yang *et al.* 2010). The details of the approach are given elsewhere (Yang *et al.* 2010); briefly, the proposed approach can be explained in a stepwise manner as follows:

Step 1: Consider a given class of chemicals (e.g., volatile organic chemicals (VOCs), PCBs, etc.). As shown in **Figure 6**, a BRN model can be established for a training set (10–20 members) of this class (designated as Class A) of chemicals in much the same way as described in an earlier publication for VOCs (Mayeno *et al.* 2005). When this is done, a *qualitative* BRN for this training set would have been established. A *qualitative* BRN contains the predicted metabolic pathways for each member of the training set for Class A chemicals, interconnections between these pathways, and metabolites and subpathways in common.

Step 2: Next, enzyme kinetic studies are conducted using commercially available recombinant human metabolic enzymes known to be involved in the metabolism of the chemicals in the training set of Class A chemicals (**Figure 7**). The purpose of such studies is to generate reaction rate constants to be incorporated into BRN modeling for generation of the *quantitative* RN. A *quantitative* BRN contains predictions for the time rates of change of the concentrations of all chemicals comprising the network.

Step 3: Using quantitative structure–activity relationship (QSAR) modeling and other computational techniques (e.g., molecular modeling and computational quantum chemistry), the reaction rate constants of chemicals other than the training set in Class A are calculated (**Figure 8**). At this stage, the generation of qualitative and quantitative BRN for Class A chemicals is possible.

Step 4: By integrating a generic PBPK model and BRN model for Class A chemicals, pharmacokinetic information for toxicologically relevant species produced from the chemicals in Class A can be predicted. This modeling effort is best

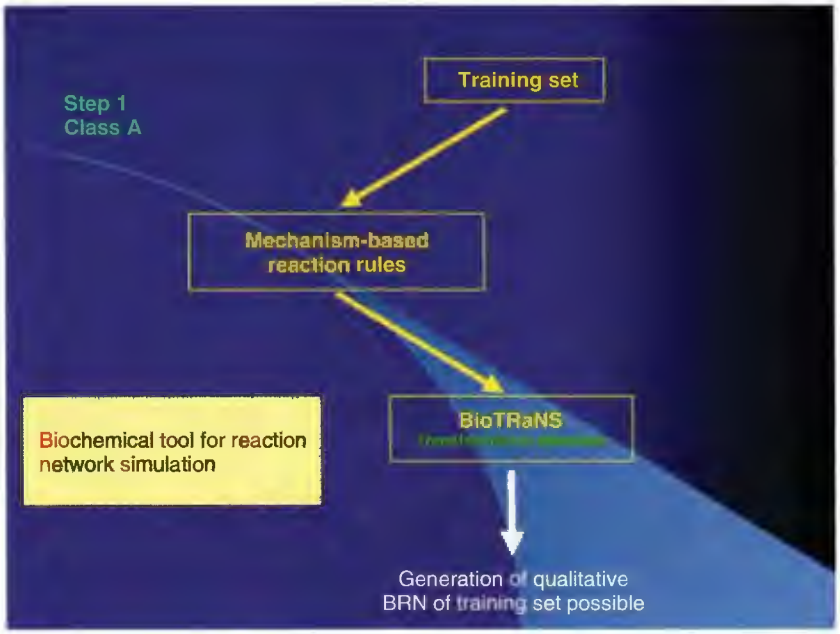

Figure 6 BRN modeling of a training set for a hypothetical class of chemicals: Generation of qualitative BRN.

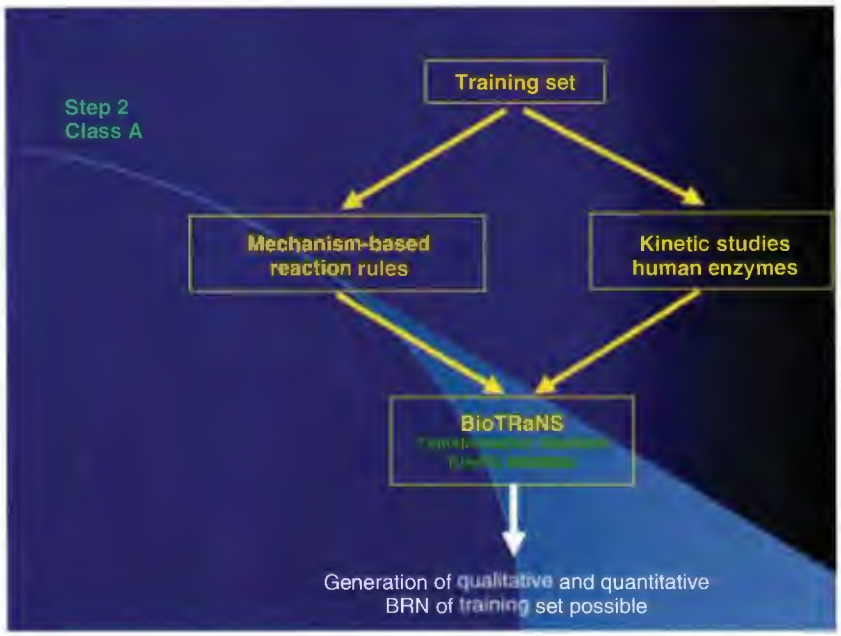

Figure 7 BRN modeling of a training set for a hypothetical class of chemicals: Generation of qualitative and quantitative BRN.

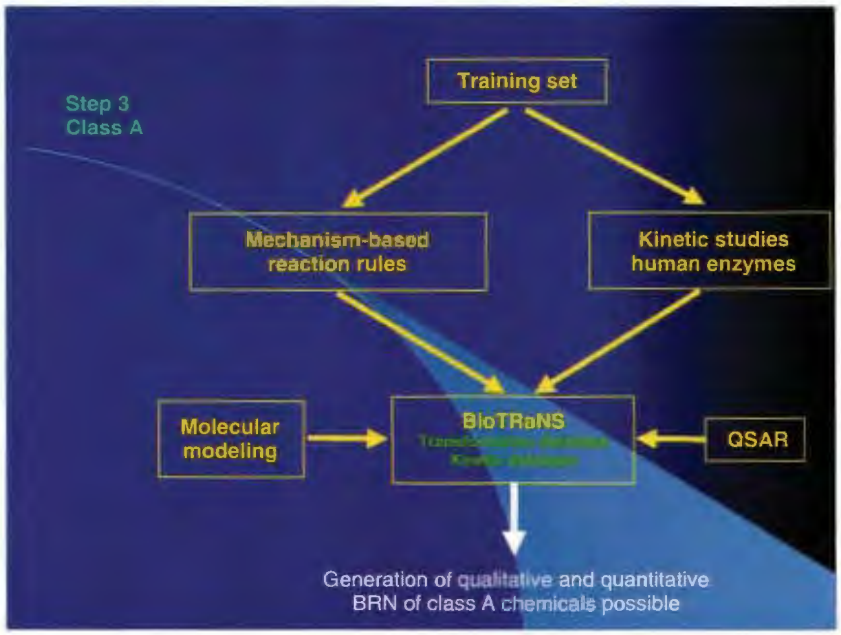

Figure 8 BRN modeling of a hypothetical class of chemicals: Generation of qualitative and quantitative BRN for Class A chemicals.

carried out by an interdisciplinary team of scientists, including toxicologists, biological modelers, and chemists. In turn, such a team of scientists is in a position to be able to predict the possible outcome of toxicities for the mixture of Class A chemicals, given that the mode(s)- or mechanism(s)-of-action have been established for Class A chemicals and their metabolites (**Figure 9**). Likewise, risk assessment for class A chemical mixtures should be possible.

Step 5: Once predictions for Class A chemicals are substantiated and the methodology validated, similar studies for other classes (Classes B, C, and D, as shown in **Figure 10**) of chemicals should be possible (**Figure 10**), thus paving the way to better understand the toxicities of a wide variety of chemical mixtures.

One of the most significant developments in advancing the science of chemical mixture toxicology is the USEPA's decision in developing cumulative risk assessment. For most of its history, the USEPA assessed risks based on individual contaminants and often focused on one source, pathway, or adverse effect. But in reality, the public is exposed to multiple contaminants from a variety of sources, and tools are needed to understand the resulting combined risks.

On 3 July 1997, the USEPA Administrator, Carol Browner, and Deputy Administrator, Fred Hansen, jointly issued a memorandum entitled 'Cumulative Risk Assessment Guidance – Phase I Planning and Scoping' to top USEPA officials. The content of this memo, quoted below, provided the essence of the reasoning for cumulative risk assessment.

> ...As you are aware, the processes that EPA and others follow to assess environmental risk are of great interest to environmental professionals and to the public, and growing attention is being given to the combined effects of multiple environmental stressors. Consistent with this, EPA and others are asking more questions about the wider and more complex issues that define a cumulative approach to risk assessment. Today, we are providing guidance for all EPA offices on cumulative risk assessment. This guidance directs each office to take into account cumulative risk issues in scoping and planning major risk assessments and to consider a broader scope that integrates multiple sources, effects, pathways, stressors and populations for cumulative risk analyses in all cases for which relevant data are available. This assures a more consistent and scientifically complete Agency-wide

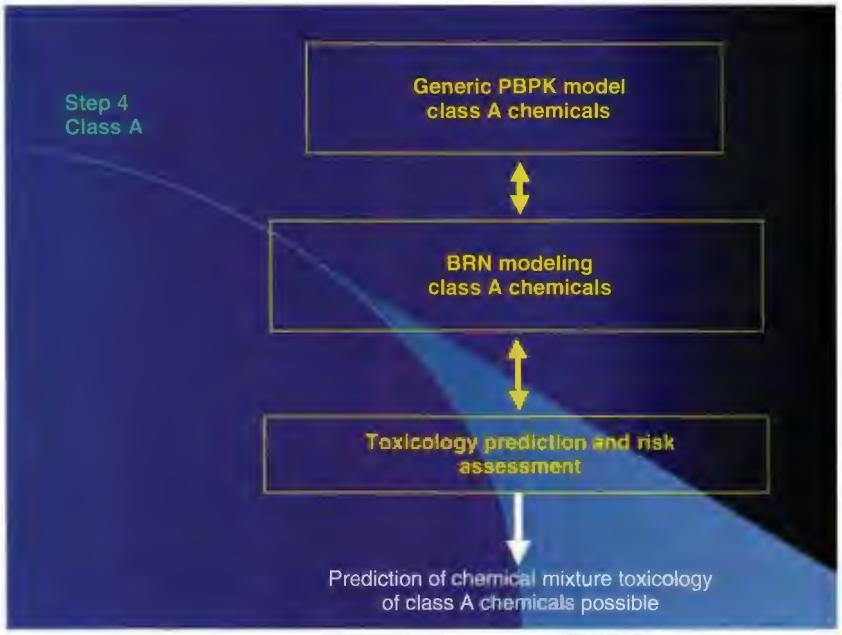

Figure 9 Integration of PBPK and BRN modeling of Class A chemicals and expert scientific assessment: Prediction of chemical mixture toxicology of Class A chemicals.

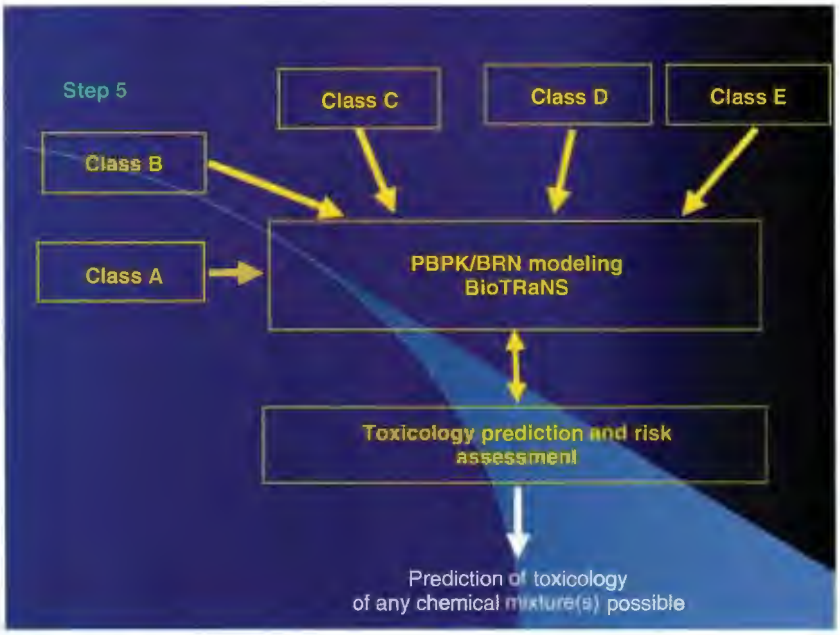

Figure 10 Prediction of toxicology for any chemical mixture(s).

approach to cumulative risk assessments in order to better protect public health and the environment. This approach provides a platform for significant advances in our scientific approach to assessing environmental risks. For most of our history, EPA has assessed risks and made environmental protection decisions based on individual contaminants – such as lead, chlordane, and DDT – with risk

assessments for these chemicals often focused on one source, pathway or adverse effect. Today, better methods and data often allow us to describe and quantify the risks that Americans face from many sources of pollution, rather than by one pollutant at a time. We are increasingly able to assess not simply whether a population is at risk, but how that risk presents itself. In addition, we are better able in many cases to analyze risks by considering any unique impacts the risks may elicit due to the gender, ethnicity, geographic origin, or age of the affected populations. Where data are available, therefore, we may be able to determine more precisely whether environmental threats pose a greater risk to women, children, the elderly, and other specific populations, and whether a cumulative exposure to many contaminants, in combination, poses a greater risk to the public.

Of particular importance are the right-to-know implications of this guidance, which requires that we build opportunities for citizens and other stakeholders to understand our ongoing risk assessments, and to provide us with their comments. Our goal is to ensure that citizens and other stakeholders have an opportunity to help define the way in which an environmental or public health problem is assessed, to understand how the available data are used in the risk assessment, and to see how the data affect decisions about risk management. Some Regions and Programs within the Agency are already making significant efforts to use integrated or cumulative risk assessment techniques, and this guidance both reflects those practices and makes them consistent across the Agency. The scope of integrated risk assessments often involves coordination across many program offices and statutory mandates for risk analysis; for example, those called for under the new safe drinking water and food safety laws. Therefore, this guidance calls for ongoing communication among risk assessors, risk managers, economists, engineers, and other technical experts within the Agency.

While we can more consistently take into account many new factors in this approach to risk assessment, many other potentially important factors are more difficult to include in our analyses, particularly the social, economic, behavioral or psychological factors that also may contribute to adverse health effects. These include, among others, such factors as existing health conditions, anxiety, nutritional status, crime and congestion. Assessment of these factors is often hampered by a lack of data to establish plausible cause-and-effect relationships; difficulties in measuring exposure, incidence and susceptibilities related to these risks; and few methods for assessing or managing these risks. This guidance does not address these factors. We expect, nonetheless, that this guidance will be updated as our understanding and experience develop; and, the Agency is focusing its research to improve our ability to incorporate these broader concerns into our cumulative risk assessments as new data and methods are brought forward.

Please take the steps needed to ensure that all major risks assessments undertaken in your area embrace this cumulative approach, so that we can better advise all citizens about the environmental and public health risks they face, and improve our ability to protect the environment and public health for the nation.

The Office of Pesticide Programs (OPP), USEPA, took the lead and conducted cumulative risk assessment on OP pesticides under the Congressional mandate of the Food Quality Protection Act (FQPA) (USEPA 2002a,b). The proposed approach contains a 10-step process: (1) Identify common mechanism group (CMG); (2) Identify potential exposures; (3) Characterize and select common mechanism endpoint(s); (4) Determine the need for a comprehensive cumulative risk assessment; (5) Determine candidate cumulative assessment group (CAG); (6) Conduct dose–response analyses and determine relative potency and points of departure; (7) Develop detailed exposure scenarios for all routes and durations; (8) Establish exposure input parameters; (9) Conduct final cumulative risk assessment; and (10) Conduct characterization of cumulative risk.

The major limitation of the current approach is the lack of consideration of toxicological interactions. In the 'Guidance on Cumulative Risk Assessment of Pesticide Chemicals That Have a Common Mechanism of Toxicity' (USEPA 2002a), it was assumed that at lower levels of exposure typically encountered environmentally no chemical interactions are expected (i.e., simple additivity). For additivity to hold true, a further assumption must be that all the common mechanism chemicals behave the same pharmacokinetically and pharmacodynamically (i.e., having the same PK and PD) (USEPA 2002a). In reality though, a case study of cumulative risk assessment of 33 OP pesticides provided BMDL (lower bound benchmark dose at ED_{10}) with a range of 3977- to 5528-fold difference between the highest BMDL for malathion to the lowest BMDL for

dicrotophos (USEPA 2002b). These 3–4 orders of magnitude differences among 'common mechanism chemicals' suggest strongly that the PK and PD are not the same among these chemicals. Thus, the probability of toxicological interactions at the level of PK and PD exists.

1.09.7 Future Perspectives: Nanotoxicology and Its Relevance to Chemical Mixtures

The advancement of nanotechnology in the twenty-first century probably represents yet another phase of industrial revolution. It was estimated that in a few years the worldwide commerce involving nanomaterials will reach $1 trillion (Hardman 2006). Presently, more than 300 commercial products are known to contain nanomaterials (Maynard *et al.* 2006). Because these nanoparticles are invisible, usually under 100 nm in diameter, and nothing much is known about their toxicities, there has been concern about health effects in humans (Maynard *et al.* 2006). Many of these nanomaterials have a core which consists of a number of metals (Hardman 2006; Nel *et al.* 2006). Thus, we are dealing with chemical mixtures. Since nanomaterials have some unique physico-chemical properties, some of them have rather persistent tissue pharmacokinetics (Lin *et al.* 2008; Yang *et al.* 2007). In one of the first published PBPK modeling papers on a nanoparticle, Quantum Dot 705 (QD705) in mice, Lin *et al.* (2008) pointed out that such unique and worrisome pharmacokinetic properties of nanoparticles might have a silver lining. Thus, while the persistence of QD705 specifically in the spleen, kidney, and liver for up to six months experimental duration was of health concern, the affinity of these nanoparticles toward these tissues might be exploited to design drug delivery systems for potential targets in these tissues. Collectively speaking, the unique properties of these nanomaterials will undoubtedly present a very important challenge for the scientists in the areas of environmental and occupational toxicology and risk assessment in the years to come.

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