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ER ID NO. 58928 ***Date Received:*** 8/20/98 ***Processor:*** YCA ***Page Count:*** 19

Privileged: (Y/N) N ***Record Category:*** P ***Record Package No:*** 0

FileFolder: N/A

Correction: (Y/N) N ***Corrected No.*** 0 ***Corrected By Number:*** 0

Administrative Record: (Y/N) Y

Refilmed: (Y/N) N ***Old ER ID Number:*** 0 ***New ER ID Number:*** 0

Miscellaneous Comments:

REFERENCE CITED

3019

THIS FORM IS SUBJECT TO CHANGE. CONTACT THE RPF FOR LATEST VERSION. (JUNE 1997)

REGULATORY TOXICOLOGY AND PHARMACOLOGY 22, 76–94 (1995)

AUG 20 1998
58928

Chemical Mixtures: Current Risk Assessment Methodologies and Future Directions

JENNIFER SEED,[1] RONALD P. BROWN,[2] STEPHEN S. OLIN, AND JEFFERY A. FORAN

International Life Sciences Institute, Risk Science Institute, 1126 16th Street N.W., Washington, DC 20036

Received March 20, 1995

Some of the most challenging problems that toxicologists confront are determining how biological effects of components in a complex mixture may interact, determining how these interactions affect the overall toxicity of the mixture, and determining how to incorporate this information into risk assessments of chemical mixtures. There has been considerable effort in this area since the publication of the U.S. Environmental Protection Agency's guidelines for risk assessment of chemical mixtures in 1986. This paper reviews the terminology used to describe chemical interactions and the methodologies that have been developed for conducting risk assessments of chemical mixtures. Particular attention is directed towards an examination of the applicability and validity of the methods for the assessment of risk posed by exposure to environmentally relevant concentrations of chemical mixtures. Limited, yet compelling, data are reviewed that suggest that for noncancer endpoints, adverse effects are unlikely to occur when the individual components in the mixture are present at levels well below their respective thresholds. Synergistic or antagonistic effects, not readily predicted from the mechanisms of action of the individual components, are possible when the mixture components are present at levels equal to or above their individual thresholds. Finally, synergistic carcinogenic effects have been observed in animal studies of mixtures, even at relatively low doses. © 1995 Academic Press, Inc.

INTRODUCTION

Humans are continuously exposed to low levels of chemical mixtures by a variety of routes and for varying lengths of time. Interactions may occur among chemicals in the mixture that alter their toxicity; therefore, the potential exists for adverse effects to occur due to exposure to the mixture that may be greater than, less than, or equal to the sum of the effects of the individual components of the mixture.

Historically, regulatory agencies have conducted risk assessments and set standards for individual chemicals. Recognizing that this approach may not be adequately protective, the U.S. EPA published general guidelines for risk assessment of chemical mixtures in 1986 (U.S. EPA, 1986). Since this publication was released, there has been considerable effort made to develop and refine methodologies for risk assessment of chemical mixtures. This paper reviews the existing and newly developed risk assessment methodologies for mixtures and critically examines assumptions in these approaches. In particular, the assumptions of dose- and response-additivity for noncarcinogens and carcinogens, respectively, are examined in light of new data on the effects of low dose exposure to chemical mixture components.

A number of graphical and statistical methods have been developed that serve as valuable tools for determining whether an interaction is occurring, and, if so, what the nature of the interaction is. However, the use of these methods for risk assessment of complex mixtures is limited for several reasons. First, these methods have been developed for relatively "simple" mixtures containing a small number of components. Second, many data points are usually required, and for this reason, the methods are impractical for most toxic effects. Finally, these methods test whether an interaction is occurring within the specific experimental dose range. Extrapolation beyond this range is not possible; therefore a quantitative estimate of risk outside the experimental range cannot be achieved. Thus, a detailed description of these methods is not included in this review.

Over the years, a number of different terms have been introduced to describe chemical interactions. The use of different terms to describe interactions has led to some confusion in the literature. The term "interaction" implies that one agent affects the biological action of

[1] To whom correspondence should be addressed.
[2] Present address: Center for Devices and Radiological Health (HFZ-112), U.S. Food and Drug Administration, 12709 Twinbrook Parkway, Rockville, MD 20857.

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TABLE 1
Terminology for Chemical Interactions

Term	Definition	Synonyms
Additivity	The combined effect of two agents is equal to the sum of the effects of each agent given alone	Summation Independence Indifference Zero interaction Simple similar action Unconditional independence
Synergism	The combined effect of two agents is greater than the sum of the effects of either agent given alone	Potentiation Coalitivity Interaction Unisynergism Augmentation Sensitization Supra-addition Independent synergism Dependent synergism Degradative synergism Greater than additive Unisynergism Cosynergism Superaddition Conditional independence
Antagonism	The combined effect of two agents is less than the sum of each agent given alone	Interaction Depotentiation Desensitization Infra-addition Negative synergy Less than additive Subaddition Inhibition Antergism Competitive antagonism Noncompetitive antagonism Uncompetitive antagonism Acompetitive antagonism

Note. Source: Calabrese (1991).

another agent. Two basic types of interactions, synergism and antagonism, can be defined (Calabrese, 1991). A synergistic effect occurs when the combined effect of two agents is greater than the effects of each agent given alone, while an antagonistic effect occurs when the combined effect of two agents is less than the sum of the effects of each agent given separately. It is important to recognize that these terms do not describe the mechanism of interactions or the quantitative aspects of the interaction; the terms simply describe whether the outcome is different from what would be expected if the agents were administered individually. Alternatively, one agent may not affect the biological action of another agent, and in such cases the term "noninteraction" would apply. An example of noninteraction is additivity; an additive effect occurs when the combined effect of two agents is equal to the sum of the effects of each agent given alone.

A list of terms that are frequently used as synonyms for synergism, antagonism, and additivity is provided in Table 1. Three of the common terms for synergism, cosynergism, potentiation, and coalitivity, are used to describe particular synergistic interactions. Cosynergism occurs when two agents enhance the toxicity of each other. Potentiation occurs when one agent enhances the toxicity of another agent, but does not affect the toxic endpoint itself. Coalitivity occurs when the combination of two agents is very toxic, whereas the action of the agents individually is ineffective. For the purposes of this review, the terminology outlined by Calabrese (1991) will be used, and wherever possible, three terms will be relied upon: synergism, antagonism, and additivity.

METHODOLOGIES FOR CHEMICAL MIXTURES RISK ASSESSMENT

In 1986, the U.S. EPA developed a framework for conducting risk assessments of chemical mixtures (U.S. EPA, 1986; Mumtaz and Hertzberg, 1993; Mumtaz *et al.*, 1993). This framework described three methods that could be used to conduct a quantitative risk assess-

ment depending on the availability of data on the mixture and on the components of the mixture. The first and most preferable method is to use data obtained from testing the actual mixture to assess the potential for health effects associated with exposure to the mixture. These data are used in the risk assessment as if the mixture was a single agent. However, this method has been used to assess the risk of relatively few mixtures due to the lack of data on the specific mixture of regulatory concern. With the exception of diesel exhaust (Albert *et al.,* 1983; Lewtas, 1985) and some mixtures of groundwater contaminants (Germolec *et al.,* 1989; Chapin *et al.,* 1989; Hong *et al.,* 1992; Heindel *et al.,* 1994; Simmons *et al.,* 1994), few studies have been conducted on well-defined chemical mixtures.

An important limitation in the use of information on the specific mixture for risk assessment is that complex mixtures can undergo changes with time, and often vary in composition from source to source. For example, in assessing the toxicity of a complex mixture such as diesel exhaust, the toxicity of a particular sample may be assessed. However, chemical interactions may occur among the components in the sample, altering its composition; therefore a sample tested at one point in time may differ from a sample tested later in time. In addition, the exact composition of complex mixtures such as diesel exhaust will vary depending on the source, which may also affect the toxicity. These factors may limit the utility of data derived from the specific mixture for risk assessment purposes.

The second most preferable method outlined by the U.S. EPA is to use data obtained from testing one or more mixtures of composition similar to the mixture of interest. A similar mixture is defined as "a mixture having the same components but in slightly different ratios, or having several common components but lacking in one or more components, or having one or more additional components." The obvious challenge in the use of this method comes in determining how similar a surrogate mixture is to the mixture of interest. The degree of similarity is evaluated on a case-by-case basis and may be based on several factors including test data and chemical and biologic properties.

Use of the "similar mixture" method requires the availability of *in vivo* data on the similar mixture so the toxicity or carcinogenic potency of the mixture of interest can be estimated. However, *in vivo* toxicity or carcinogenicity data are available for relatively few complex mixtures. One alternative that may be used when assessing the carcinogenic potential of several mixtures in a particular class is the relative potency or comparative potency method based on *in vitro* data (Albert *et al.,* 1983). When the relationship between *in vitro* data and *in vivo* data can be established for one mixture, it is theoretically possible to estimate the *in vivo* carcinogenic potency of similar mixtures based on the results of *in vitro* assays. This method has been used to estimate the cancer risk of several diesel emissions (Albert *et al.,* 1983; Lewtas, 1985), but it has not received enough further use to determine its widespread applicability.

The lack of quantitative data on all but a few chemical mixtures limits the use of the methods described above. The third method that the U.S. EPA recommends for conducting a risk assessment of chemical mixtures, the "component-based" method, is therefore used most often. This method involves assessing the risk posed by exposure to the individual components of the mixture. Data from risk assessments of individual compounds are then used to estimate the risk of the mixture of interest by applying a dose additivity model for systemic toxicants and a response additivity model for carcinogens.

Dose additive approaches are based on the assumption that the components of a mixture behave as if they were dilutions or concentrations of each other. It is also assumed that the mixture components act via a common toxicological mechanism. The two most accepted dose additive approaches are the hazard index (HI) approach and the toxicity equivalency factor (TEF) approach. In both approaches, the doses of the mixture components are first normalized to a common scale and then summed. In the HI approach, the doses are standardized using health-based values such as acceptable daily intakes (ADIs) or reference doses (RfDs). In the TEF approach, the dose of each component of the mixture is normalized against the dose of one of the components, usually the most potent compound, to derive a relative potency for each. The relative potencies are then summed to estimate the toxic or carcinogenic potency of the mixture. These approaches, as well as methods to refine them, are described below.

Hazard Index Approach

The first step in the hazard index approach involves calculation of the hazard quotient of each compound in the mixture which is equal to the dose of the compound divided by the maximum acceptable dose for that compound, such as an RfD. The hazard quotients for each mixture component are then added together to calculate the HI as follows:

$$\mathrm{HI} = \sum_{i=1}^{n} \frac{\mathrm{dose}_i}{\mathrm{RfD}_i},$$

where n is the number of components of the mixture (U.S. EPA, 1986; Mumtaz and Hertzberg, 1993; Mumtaz *et al.,* 1993).

This method is most appropriately used for mixture components that induce the same toxic effect in the same organ system via the same mechanism of action since dose additivity is assumed to be valid only under these conditions. Although it may be possible to make

this assumption for relatively simple mixtures of chemically or toxicologically related substances, the assumption may be violated for complex mixtures of compounds that act on multiple organ systems by various mechanisms. Multiple mechanisms and target organs are more likely when dealing with complex mixtures such as contaminated groundwater or soil at a hazardous waste site. In these situations, the U.S. EPA recommends that a separate hazard index be calculated for each toxic endpoint of concern.

The hazard index should be viewed as an approximation of the noncarcinogenic risk posed by exposure to the mixture and not as an absolute risk value. This concept is explained in the U.S. EPA (1986) guidelines for conducting chemical mixtures risk assessments:

> The hazard index provides a rough measure of likely toxicity and requires cautious interpretation. The hazard index is only a numerical indication of the nearness to acceptable limits of exposure or the degree to which acceptable exposure levels are exceeded.

There is considerable debate regarding the values for the hazard index that represent an unreasonable risk. Generally, it is assumed that there may be increased risk when the hazard index is greater than 1. However, factors such as the sensitivity of the endpoint and size of the uncertainty factor used to derive the hazard quotient for each compound should be taken into account in deciding whether a hazard index represents an unreasonable risk.

Although the lack of data on specific interactions requires that the additivity assumption be invoked for most chemical mixtures, the U.S. EPA guidelines encourage the use of data on interactions (synergism, antagonism) when available. Several modifications of the standard hazard index approach have been proposed to incorporate data on interactions. For example, the Subcommittee on Mixtures of the NRC Safe Drinking Water Committee (NRC, 1989) recommended using an additional uncertainty factor, when appropriate, to derive the hazard index for a mixture to protect against potential synergistic interactions. The hazard index would then be calculated as:

$$\mathrm{HI} = (\mathrm{UF}) \sum_{i=1}^{n} \frac{\mathrm{dose}_i}{\mathrm{RfD}_i} .$$

The NRC subcommittee urged that such an approach be flexible and recommended the use of uncertainty factors ranging from 1 to 100. The value of the uncertainty factor should reflect two issues: First, the uncertainty factor should reflect the confidence in the available information on the interaction; second, since the potential for synergistic responses increases as the dose or number of mixture components increases (NRC, 1989), the uncertainty factor should reflect the concentration of the mixture components. For example, an uncertainty factor of 1, which is equivalent to the additivity assumption, would be used when there are data indicating that a synergistic response is unlikely or when the concentration of the mixture components is low. An uncertainty factor of 10 would be used when less is known about the potential for interactions to occur or if relatively high concentrations of the mixture components are present.

While the potential exists for synergistic interactions to occur among components of a complex mixture, it is also possible that antagonistic responses will be observed. Since the approach proposed by the NRC (1989) only incorporates additive and synergistic responses, it may overestimate the risk from exposure to some complex mixtures, especially those containing low levels of constituent compounds. A different scheme has recently been developed by Mumtaz and Durkin (1992) to account for both synergistic and antagonistic interactions in the derivation of the hazard index. In this approach, an alphanumeric weight-of-evidence classification is assigned to a binary mixture that takes into account a number of factors including whether that interaction is likely to be additive, antagonistic, or synergistic; an understanding of the mechanism by which the interaction may occur; the potential toxicological significance of the interaction; the duration and sequence of exposure; whether *in vitro* or *in vivo* data were used to describe the interaction; and whether the toxicity data were collected using a route of exposure similar to the route expected under environmentally relevant situations. Numbers or letters are ascribed to each of these factors as shown in Table 2.

The features of specific interactions can be characterized using this method. For example, selenium is known to inhibit the toxic effects of cadmium upon coexposure. Features that would be important in the weight-of-evidence classification of this interaction include: (1) the mechanism by which this interaction occurs is fairly well understood; (2) data from other related compounds are consistent with the proposed mechanism; (3) the toxicological significance of this interaction can be demonstrated; and (4) *in vivo* data on this interaction are available from long-term studies that utilized the same route by which humans are likely to be exposed. Using the letters and numbers that correspond to each of these features (Table 2), an alphanumeric weight-of-evidence classification for the effect of selenium on the toxicity of cadmium can be represented as <II.A.1.a.i.

Mumtaz and Durkin (1992) have shown how this classification scheme can be used to provide a qualitative and quantitative estimate of the effect of interactions on the aggregate toxicity of mixtures occurring at hazardous waste sites. Assuming binary compound interaction data are available for many of the compounds present at the site, a matrix can be formed containing the alphanumeric weight-of-evidence classi-

TABLE 2
Classifications Used in the Weight-of-Evidence Scheme

Determine if the interaction of the mixture is additive (=), greater than additive (>), or less than additive (<)[a]

Classification of Mechanistic Understanding

I. Direct and Unambiguous Mechanistic Data:
The mechanism(s) by which the interactions could occur has been well characterized and leads to an unambiguous interpretation of the direction of the interaction.

II. Mechanistic Data on Related Compounds:
The mechanism(s) by which the interactions could occur is not well characterized for the compounds of concern but structure activity relationships, either quantitative or informal, can be used to infer the likely mechanisms and the direction of the interaction.

III. Inadequate or Ambiguous Mechanistic Data:
The mechanism(s) by which the interactions could occur have not been well characterized or information on the mechanism(s) do not clearly indicate the direction that the interaction will have.

Classification of Toxicologic Significance

A. The toxicologic significance of the interaction has been directly demonstrated.
B. The toxicologic significance of the interaction can be inferred or has been demonstrated in related compounds.
C. The toxicologic significance of the interaction is unclear.

Modifiers

1. Anticipated exposure duration and sequence.
2. A different exposure duration or sequence.
   a. *In vivo* data
   b. *In vitro* data
      i. The anticipated route of exposure
      ii. A different route of exposure

Note. Source: Reproduced, with permission, from Mumtaz and Durkin (1992).
[a] In this classification scheme, Mumtaz and Durkin (1992) use the terms less-than-additive to describe antagonistic effects and greater-than-additive to describe synergistic effects.

fications for each of the binary mixture pairs. An example of such a matrix is shown in Fig. 1.

Using this approach, it may be possible to draw some qualitative conclusions about the likelihood and significance of the interactions that may occur among the mixture components. For example, the matrix may indicate that antagonistic interactions predominate, so it is possible that a hazard index derived in the conventional fashion may overestimate the risk upon exposure to the mixture. To provide a semiquantitative estimate of the likelihood that the risk from exposure to a mixture will be greater than or less than the sum of the hazard quotients of the mixture components, the alphanumeric classifications are converted to numeric val-

		AFFECTED BY					
		1. Cd	2. CCl_4	3. $CHCl_3$	4. Se	5. Tol.	6. Xyl.
AFFECTS	1. Cadmium		<I. A.2.	<I. B.2.	<III. C.1.	>II. B.1.	>II. B.1.
	2. Carbon tetrachloride	?		>III. A.2.	?	>II. B.1.	>II. B.1.
	3. Chloroform	?	>III. A.2.		?	>II. B.2.	>II. B.1.
	4. Selenium	<II. A.1.	<I. B.1.	<I. B.1.		>II. B.2. b.ii.	>II. B.2. b.ii.
	5. Toluene	?	>II. B.2.	>II. B.2.	?		=1. B.2.
	6. Xylene	?	>II. B.2.	>II. B.2.	?	=1. B.2.	

FIG. 1. Qualitative interaction matrix for example compounds. Source: Reproduced, with permission, from Mumtaz and Durkin (1992).

TABLE 3
Data Quality Weighting Factors for Weight-of-Evidence Scheme

Determine if the interaction of the mixture is additive (D = 0), greater than additive (D = 1), or less than additive (D = −1)[a]	
Mechanistic Information	*Weight*[b]
I. Direct Mechanistic Data	1.0
II. Mechanistic Data on Related Compounds	0.71
III. Inadequate or Ambiguous Mechanistic Data	0.32
Toxicological Significance	
A. Interaction directly related to toxicity	1.0
B. Significance of interaction can inferred	0.71
C. Significance of interaction unclear	0.32
Modifiers	
1. Duration/sequence of concer	1.0
2. Different duration/sequence	0.79
a. *In vivo* data	1.0
b. *In vitro* data	0.79
i. For the route of exposure	1.0
ii. For a different route of exposure	0.79

Note. Source: Reproduced, with permission from Mumtaz and Durkin (1992).

[a] In this classification scheme, Mumtaz and Durkin (1992) use the terms less-than-additive to describe antagonistic effects and greater-than-additive to describe synergistic effects.

[b] Maximum possible weight = 1.0; minimum possible weight = 0.05.

ues. The numeric weights corresponding to each alphanumeric category are shown in Table 3.

A direction factor of 0, −1, or 1 is also assigned depending on whether the interaction is additive, antagonistic, or synergistic, and a weighting factor is calculated from the product of the ratings from the other alphanumeric classification components. In the example described earlier for the interaction of selenium and cadmium, the alphanumeric classification assigned to this combination of <II.A.1.a.i would yield a direction factor of −1 and a weighting factor of [0.71 × 1.0 × 1.0 × 1.0 × 1.0] or −0.71. Using these weight-of-evidence values, an interaction factor can be derived for each binary mixture. The overall weight-of-evidence of risk posed by exposure to the complex mixture can then be represented by the sum of the interaction factors for each binary mixture. The weight-of-evidence value is then used to adjust the hazard index.

Although the weight-of-evidence approach proposed by Mumtaz and Durkin (1992) is conceptually attractive because it allows one to incorporate data on the nature of the interaction into the assessment, its use may be limited by several factors. First, not only the magnitude but the very nature of the interaction occurring between two compounds may differ depending on the dose of the compounds and species of animals used in the study, the route of exposure, and the temporal relationship between administration of one mixture component and the others. Therefore, it is possible for a given binary mixture to have different "direction factors" depending on the conditions of the study. Second, inherent in the weight-of-evidence approach is the assumption that the risk posed by exposure to a complex mixture can be estimated by the sum of the interactions occurring between pairs of complex mixture components. However, the effect of one compound on the toxicity of another compound in the mixture may be modified by the presence of the other components of the mixture. For example, it can be demonstrated that selenium antagonizes the toxic effects of cadmium, as discussed above. However, it is unclear how selenium would affect the toxicity of cadmium in the presence of other compounds. Third, it is likely that interaction data will not be available for many of the possible binary mixture pairs.

The uncertainties inherent in this approach require that it be used with caution. While derivation of a hazard index using the conventional approach is admittedly imprecise and probably overly conservative, it does not purport to provide an accurate characterization of risk. Therefore, the potential advantages in "fine tuning" the risk estimates provided by the hazard index approach by using a weight-of-evidence procedure may be outweighed by the uncertainties associated with this approach. Nevertheless, it does represent an interesting concept and testing and refinement of this approach should be encouraged where data are available.

Toxicity Equivalency Factor Approach

A modification of the dose-additive approach, known as the TEF approach, has been used to estimate the toxic or carcinogenic potency of complex mixtures of toxicologically and structurally related compounds in certain chemical classes for which there are insufficient *in vivo* toxicity data for individual members of the class. In this approach, the potency of the components of a mixture in short-term testing is compared to the potency of a compound that has undergone both short-term and chronic studies. The relative potencies of the mixture components in the short-term tests are assumed to be identical to their relative potencies after long-term exposure. Relative potency is represented by a TEF assigned to each compound. The product of the level of the compound in a mixture and its TEF is known as a toxicity equivalent. The sum of the toxicity equivalents for each mixture component is assumed to provide an estimate of the toxic or carcinogenic potency of the mixture.

The concept of TEFs was developed as an interim measure to assess the toxicity of complex mixtures of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs). There was a wealth of toxicity data for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), but limited data for the other environmentally relevant congeners. Congeners that could be included in a TEF-

TABLE 4
Relative Range of Toxicities of PCDD and PCDF Congeners Compared to That of 2,3,7,8-TCDD

Congener	*In vivo* responses	*In vitro* responses
2,3,7,8-tetraCDD	1.0	1.0
1,2,3,7,8-pentaCDD	0.59–0.053	0.64–0.07
1,2,3,6,7,8-hexaCDD	0.16–0.0152	0.5–0.005
1,2,3,7,8,9-hexaCDD	0.14–0.016	0.009
1,2,3,4,7,8-hexaCDD	0.24–0.015	0.13–0.015
1,2,3,4,6,7,8-heptaCDD	0.0076	0.003
octaCDD	<0.0013	0.0006
2,3,7,8-tetraCDF	0.17–0.16	0.43–0.006
1,2,3,7,8-pentaCDF	0.9–0.018	0.13–0.003
2,3,4,7,8-pentaCDF	0.8–0.12	0.67–0.11
1,2,3,4,7,8-hexaCDF	0.18–0.038	0.2–0.013
1,2,3,6,7,8-hexaCDF	—	0.48–0.037
1,2,3,7,8,9-hexaCDF	—	—
2,3,4,6,7,8-hexaCDF	0.097–0.017	0.1–0.015
1,2,3,4,6,7,8-heptaCDF	0.22	—
1,2,3,4,7,8,9-heptaCDF	0.20	—
octaCDF	—	—

Note. From Safe (1993).

scheme had to meet several criteria including: (1) structural similarity to 2,3,7,8-TCDD, (2) binds to the Ah-receptor, (3) elicits dioxin-specific biochemical and toxic responses, and (4) is persistent and accumulates in the food chain (Barnes *et al.*, 1991). These criteria are met by the PCDDs, PCDFs, and some coplanar polychlorinated biphenyls (PCBs).

The first step in deriving TEFs is to identify relative potency values, or a range of values, for each structurally or toxicologically related compound in the class. The potency values for each compound are derived by comparing the toxic or carcinogenic potency of a particular compound in the class to another compound in that class that has either undergone extensive toxicity testing and/or is the most potent representative of that class. For PCDDs and PCDFs, 2,3,7,8-TCDD meets both of these criteria. As a result, the relative potency of every other compound in the class is based on the potency of 2,3,7,8-TCDD, with 2,3,7,8-TCDD assigned a relative potency value of 1. Relative potency values for PCDDs and PCDFs have been based on numerous *in vitro* and *in vivo* studies. The range of relative values for various members of these classes is provided in Table 4.

Based on the relative potency values, TEFs can be assigned to each compound. Regulatory agencies generally take a conservative approach and assign a TEF for each compound that corresponds to the upper bound values in the range of relative potencies. In addition, more weight is given to data from chronic bioassays and reproductive toxicity studies in assigning a TEF, if such data are available. The TEFs assigned to PCDD and PCDF congeners by various authorities are listed in Table 5. The TEF of each PCDD and PCDF congener is then multiplied by the concentration of each congener in the mixture to yield the toxicity equivalent (TEQ). The toxicity or carcinogenicity of the mixture is then estimated from the sum of the TEQs.

The TEF approach has been fairly successful in assessing the toxicity of mixtures of PCDDs and PCDFs. For example, Eadon *et al.* (1986) characterized the composition of an extract of soot that contained a complex mixture of PCDDs and PCDFs and quantified the amount of each PCDD and PCDF congener found in the mixture. TCDD equivalents were estimated based on the concentration of the congeners and TEFs assigned to each compound. The sum of the TCDD equivalents for PCDDs and PCDFs in this mixture was 15.37 (0.82 ppm for PCDDs and 14.55 ppm for PCDFs). Toxicity studies conducted in guinea pigs with the soot extract yielded ED50 values for a series of endpoints ranging from 2 to 21 ppm, with an average value of 12.5 ppm TCDD equivalents. Therefore, the average of the experimentally observed ED50 values approximated the estimated toxicity of the mixture, based on 2,3,7,8-TCDD equivalents, quite well.

Despite the ability of the TEF approach to predict the potency of some mixtures of PCDDs, PCDFs, and PCBs, it is important to recognize that there are many limitations in the application of this approach. First, there are quantitative differences in the potency of PCDD and PCDD-like congeners between species and between responses. These differences lead to a range of relative potency values from which a congener-specific

TABLE 5
Proposed Toxic Equivalency Factors for the PCDD and PCDF Congeners

	TEFs proposed by		
Congener	Safe	International EPA	Nordic
PCDDS			
2,3,7,8-tetraCDD	1.0	1.0	1.0
1,2,3,7,8-pentaCDD	0.5	0.5	0.5
1,2,3,6,7,8-hexaCDD	0.1	0.1	0.1
1,2,3,7,8,9-hexaCDD	0.1	0.1	0.1
1,2,3,4,7,8-hexaCDD	0.1	0.1	0.1
1,2,3,4,6,7,8-heptaCDD	0.01	0.01	0.01
octaCDD	0.001	0.001	0.001
PCDFs			
2,3,7,8-tetraCDF	0.1	0.1	0.1
2,3,4,7,8-pentaCDF	0.5	0.5	0.5
1,2,3,7,8-pentaCDF	0.1	0.05	0.01
1,2,3,4,7,8-hexaCDF	0.1	0.1	0.1
2,3,4,6,7,8-hexaCDF	0.1	0.1	0.1
1,2,3,6,7,8-hexaCDF	0.1	0.1	0.1
1,2,3,7,8,9-hexaCDF	0.1	0.1	0.1
1,2,3,4,6,7,8-heptaCDF	0.1	0.01	0.01
1,2,3,4,7,8,9-heptaCDF	0.1	0.01	0.00
octaCDF	0.001		

Note. From Safe (1993).

TABLE 6

Estimates of Toxicity Equivalence Factors for Five Organophosphate Insecticides Using the NOAEL for Chlorpyrifos as the Reference Standard

Pesticide	NOAEL	NOAEL Ratio = TEF
Acephate	NA	NA
Chlorpyrifos	0.03 mg/kg bw/day Humans, ChE inhibition	1
Dimethoate	0.05 mg/kg bw/day Rats, ChE inhibition	0.03/0.05 = 0.6
Disulfoton	NA	NA
Ethion	0.05 mg/kg bw/day Humans, ChE inhibition	0.03/0.05 = 0.6

Note. Reprinted with permission from National Research Council (1993).

TEF is derived. Therefore, the predictive ability of the TEF approach is dependent upon the endpoint and species selected when measuring the potency of the mixture. For example, Nagao *et al.* (1993) examined cleft palate formation in NMRI mice following administration of PCDD and PCDF mixtures. The potency of the PCDD mixture was accurately predicted when I-TEFs (NATO/CCMS, 1988) were used for the PCDD congeners; however, the cleft palate-inducing potency of a PCDF mixture was overpredicted when the I-TEFs for the PCDF congeners were used. Another limitation of the TEF approach is that the TEF values are derived from short-term or subchronic *in vivo* experiments or from *in vitro* bioassays. Therefore, any differences in toxicokinetics are largely ignored. Third, only Ah-receptor-mediated effects are used in the TEF approach for PCDDs, PCDFs, and PCBs. Many of the coplanar PCBs elicit a number of effects that are not mediated by the Ah receptor (reviewed by Ahlborg *et al.*, 1992). Therefore, the TEFs will not be predictive for all toxic responses associated with these compounds. Nevertheless, this approach has been a valuable "interim" tool to assess the toxicity of mixtures of several important classes of compounds, including PCDDs and PCDFs (NATO/CCMS, 1988; Ahlborg, 1989; Ahlborg *et al.*, 1992; Bellin and Barnes, 1989; Kutz *et al.*, 1990; Safe, 1990, 1993), PCBs (Ahlborg *et al.*, 1994; Barnes *et al.*, 1991; U.S. EPA, 1987, 1991; Safe, 1990, 1993) and polycyclic aromatic hydrocarbons (Nisbet and LaGoy, 1992; U.S. EPA, 1993).

The TEF approach has also been applied to assess the toxicity of organophosphate pesticides. The NRC Committee on Pesticides in the Diets of Infants and Children (NRC, 1993) recently used a TEF approach to estimate the aggregate risk to children from dietary exposure to a mixture of pesticides. The committee examined five organophosphate pesticides including acephate, chlorpyrifos, dimethoate, disulfoton, and ethion. All five pesticides are cholinesterase inhibitors and may be present as pesticide residues on fruits and vegetables. In contrast to the situation with the PCDDs, PCDFs and PCBS, *in vivo* toxicity studies have been conducted for the five pesticides. The TEF approach was used in this case as a means of expressing the cholinesterase inhibition potency of each pesticide relative to the potency of chlorpyrifos, which the committee decided was the most potent cholinesterase inhibitor of the five. The TEF was defined as the ratio of the no-observed-adverse-effect-level (NOAEL), or lowest-observed-adverse-effect-level (LOAEL), for each pesticide to the NOAEL (or LOAEL) for chlorpyrifos, shown in Tables 6 and 7, respectively. Then, the committee used FDA residue data, which were obtained from 1988 and 1989 compliance, surveillance, import, and domestic sampling, to estimate the amount of residue of each of the five pesticides on eight foods (apples, oranges, grapes, beans, tomatoes, lettuce, peaches, and peas) and in three fruit juices (apple, orange, and grape). Residues for each pesticide were then converted to chlorpyrifos equivalents by multiplying the concentration of each pesticide on the food items by the potency values shown in Table 7; the TEFs based on the ratio of LOAELs were used since TEFs based on NOAELs were not available for two of the pesticides (Table 6).

There are several factors to keep in mind regarding the approach by the NRC committee to develop TEFs for these organophosphate pesticides. First, TEFs derived from the ratio of LOAELs were used for all five pesticides. It is apparent that somewhat different results may have been achieved if TEFs based on the ratio of NOAELs had been used. For example, a TEF of 0.6 was derived for ethion using the ratio of NOAELs (Table 6), whereas a TEF of 1.33 was derived using the ratio of LOAELs (Table 7). Second, the dose levels used to determine the LOAEL were from the "critical study," as defined by the EPA for the derivation of the RfD;

TABLE 7

Estimates of Toxicity Equivalence Factors for Five Organophosphate Insecticides Using the LOAEL for Chlorpyrifos as the Reference Standard

Pesticide	LOAEL	LOAEL Ratio = TEF
Acephate	0.12 mg/kg bw/day Rats, ChE inhibition	0.1/0.12 = 0.83
Chlorpyrifos	0.10 mg/kg bw/day Humans, ChE inhibition	0.1/0.10 = 1.0
Dimethoate	0.25 mg/kg bw/day Rats, ChE inhibition	0.1/0.25 = 0.40
Disulfoton	0.04 mg/kg bw/day Rats, ChE inhibition	0.1/0.04 = 2.5
Ethion	0.075 mg/kg bw/day Humans, ChE inhibition	0.1/0.075 = 1.33

Note. Reprinted with permission from National Research Council (1993).

the EPA defines the critical study as that study showing effects at the lowest dose level. However, in some cases (chlorpyrifos and ethion) the critical study was a human study, and in other cases (acephate, dimethoate, and disulfoton) the critical study was a rat study. Although in all cases cholinesterase inhibition was the endpoint of concern, the critical dose levels from human studies should be compared with caution with dose levels from rodent studies.

Response Additive Methods

A response additive model is generally used in cancer risk assessment of chemical mixtures (U.S. EPA, 1986; Mumtaz and Hertzberg, 1993; Mumtaz *et al.*, 1993). The principal assumption is that the components of the mixture act on the same target site, but by different mechanisms of action; therefore, the toxicological responses of the organism to each component in the mixture (rather than the doses) are summed. Response is generally expressed as risk, which is calculated as the product of dose and carcinogenic potency, usually the upper 95% confidence interval on the potency. This relationship is assumed to be valid only when the dose–response curve is linear at low doses. The risk of a mixture of compounds is estimated as the sum of the risks of the individual mixture components as follows:

$$P = \sum_{i=1}^{n} d_i B_i,$$

where d_i and B_i are the dose and potency of the ith compound, respectively, and n is the number of components in the mixture.

Chen *et al.* (1990) proposed a more formal statistical method for estimating joint risk. In this method, the upper 100(1-a)% confidence interval is calculated for each component of the mixture, and these values are summed to provide an estimate of the carcinogenic risk of the mixture. More recently, Kodell and Chen (1994) have expanded upon this approach to demonstrate how the upper confidence limit on the total risk from consumption of mixtures of drinking water contaminants can be derived using direct likelihood or bootstrap methods (Table 8). These risk estimates were based on potency values derived using the multistage model of carcinogenesis; however, the bootstrap procedure may be appropriate for other models as well, including the two-stage clonal expansion model.

As shown in Table 8, summation of the individual upper 95% confidence intervals for chlorobenzene and hexachlorobenzene would overestimate the upper-bound risk of the binary mixture of these compounds by 2–6%, depending on the method used. Similarly, the upper-bound risk for the mixture of chlorobenzene and trichloroethylene may be overestimated by 12–15%, if the total upper-bound risk for the mixture is assumed to be equal to the sum of the upper confidence levels of the individual compounds. In most cases, the magnitude of the difference in the cancer risk estimates calculated by these various methods will be greatest for mixtures of equipotent compounds. However, even for mixtures of equipotent compounds, the differences in joint risk estimated by summing the upper 95% confidence levels and estimated by the methods of Kodell and Chen (1994) are not great.

As an alternative to summing the potencies of individual compounds, modifications can be made to stochastic dose–response models to estimate the potency of a mixture of carcinogens. A variety of models are available for dose–response extrapolation including linear, multiplicative, logistic, and multistage models. The NRC Committee on Methods for the *In Vivo* Testing of Complex Mixtures (NRC, 1988) developed a generalized additive model that can be used to obtain risk estimates of a mixture of carcinogens regardless of the model used to estimate risks for the individual components of the mixture. To date, this model has only been tested with the epidemiological data of Reif (1984) who examined the individual and joint effects of tobacco smoking and uranium exposure. The model was able to predict the risk of lung cancer in individuals exposed to both cigarette smoke and uranium quite well. However, the model needs to be tested with more data sets before its widespread applicability can be assessed.

Mechanistic Models for Chemical Mixtures Risk Assessment

There have also been efforts to develop mechanistic models of carcinogenic and noncarcinogenic processes that can be used in chemical mixtures risk assessment. For example, Kodell *et al.* (1991) have suggested an application of the two-stage clonal expansion model of carcinogenesis to mixtures of compounds that may affect the same or different stages of the carcinogenic process. The two-stage clonal expansion model (also known as the Moolgavkar–Venzon–Knudsen (MVK) model) explicitly takes into account tissue growth and cell kinetics. The model is based on the hypothesis that a malignant cell is produced after two mutations of a single stem cell, and a tumor will result if the initiated stem cell goes through a process of clonal expansion. According to Kodell *et al.* (1991), the agent producing the first mutational event is known as an initiator; the second mutation is produced by a completer. Acceleration of the clonal expansion of initiated cells is mediated by promoters. Kodell *et al.* (1991) determined the expected result of this model, based on theoretical considerations, for different binary combinations of an initiator, completer, and promoter (Table 9).

Although there are several instances when exposure to compounds that affect different stages of the carcinogenic process can be expected, on theoretical

TABLE 8
Upper-95%-Confidence Limits on Extra Cancer Risk for Selected Chemicals at Concentrations of 1 μg/liter in Drinking Water

Chemical		Statistical method of UCL: Direct likelihood	Direct bootstrap	ED_{01} bootstrap
A. Chlorobenzene		2.14×10^{-7}	1.91×10^{-7}	1.91×10^{-7}
B. Hexachlorobenzene		1.92×10^{-6}	1.50×10^{-6}	1.50×10^{-6}
C. Trichloroethylene		2.74×10^{-7}	2.79×10^{-7}	2.79×10^{-7}
D. 1,1,1-Trichloroethane		3.70×10^{-8}	3.78×10^{-8}	3.78×10^{-8}
Mixture	**Mixture method**	**Direct likelihood**	**Direct bootstrap**	**ED_{01} bootstrap**
A and B	Sum UCLs	2.14×10^{-6}	1.69×10^{-6}	1.69×10^{-6}
	Proposed	2.01×10^{-6}	1.63×10^{-6}	1.65×10^{-6}
A and C	Sum UCLs	4.88×10^{-7}	4.70×10^{-7}	4.70×10^{-7}
	Proposed	4.30×10^{-7}	4.01×10^{-7}	4.03×10^{-7}
A, B, C	Sum UCLs	2.41×10^{-6}	1.97×10^{-6}	1.97×10^{-6}
	Proposed	—	1.83×10^{-6}	1.86×10^{-6}
A, B, C, D	Sum UCLs	2.45×10^{-6}	2.01×10^{-6}	2.01×10^{-6}
	Proposed	—	1.84×10^{-6}	1.88×10^{-6}

Note. Source: Reproduced, with permission, from Kodell and Chen (1994).

grounds, to yield greater-than-additive responses, it is likely that these effects will be seen only at relatively high doses. In fact, a number of cases of greater-than-additive carcinogenic responses are well documented (e.g., aflatoxin and hepatitis B; virus in liver cancer). Kodell and colleagues (1991) argue that the low-dose response to two genotoxic agents will approximate additivity, even though, from a theoretical perspective, a multiplicative relationship is expected. Further, multiplicative or supramultiplicative effects expected between an initiator and a promoter or two promoters may not occur at low doses because of a threshold for the effect of the promoter. Therefore, based on an understanding of the events represented by the clonal two-stage model of carcinogenesis, low-dose exposure to compounds that affect the various stages of the process can be modeled by using the assumption of additivity, even when theoretical principles suggest that the assumption of additivity is violated at higher doses.

TABLE 9
Interaction for Simple Exposures

Carcinogen C_1	Carcinogen C_2	Interaction
Initiator	Initiator	Additive
Completer	Completer	Additive
Initiator	Completer	Multiplicative
Promoter	Promoter	Supramultiplicative
Initiator	Promoter	Multiplicative-supramultiplicative
Initiator	Initiator and Completer	Supra-additive submultiplicative
Initiator	Initiator and Promoter	Supra-additive supramultiplicative
Promoter	Promoter and Completer	Supramultiplicative

Note. From Kodell *et al.* (1991).

Other mechanistic models also can be applied to the chemical mixtures problem for carcinogens (Cohen and Ellwein, 1991). Ultimately, the challenge will be to develop a full enough understanding of the complex biological interactions and their dose dependencies to predict carcinogenic responses over a wide range of doses.

Clevenger *et al.* (1991) have developed a pharmacodynamic model to describe the interaction occurring between a substrate and an inducer or inhibitor of the mixed function oxidase (MFO) enzyme system based on relationships between dose, elimination rate, and duration of effect. Data on the effect of piperonyl butoxide on the ability of hexobarbital to induce sleep was used to test the model. Although the model was applicable for analyzing this particular system, its general use for risk assessment purposes is limited for several reasons. First, as the model is currently written, its use is restricted to two compounds. Second, the model defines "effect" as the duration of the effect. This is a common definition in pharmacology, but an uncommon definition (or representation) in toxicology. Finally, the model is able to predict a response when the concentration of each compound is known. However, extrapolation of the response beyond the experimental range is not possible.

TOXICITY STUDIES OF COMPLEX MIXTURES ADMINISTERED AT LOW DOSES

Central to the methods described in the previous section are the assumptions of dose- and response-additivity for noncarcinogens and carcinogens, respectively. There is concern that these methods may be inadequate because potential antagonistic and synergistic interactions are not taken into account. Recently, a number of studies have been conducted to assess the toxicity of complex mixtures at environmentally relevant doses. While most of these studies do not directly assess the validity of the additivity assumption, the results have important implications regarding the likelihood that significant interactions will occur at low-dose levels.

The U.S. EPA (1986) suggested that the dose-additive approach is appropriate for noncarcinogens when mixture components are present at low doses, and when the mixture components induce the same toxic effect in the same organ system via the same mechanism of action. This seems appropriate if low dose is defined in relationship to the threshold level for noncancer endpoints. If a mixture contains a variety of compounds that act via a common mechanism, but each individual compound is present at a level slightly below, or at its threshold, an additive effect is likely to occur; however, the likelihood decreases as component concentrations decrease below their respective thresholds. In contrast, if a mixture contains a variety of compounds that do not act on a common target or by a similar mechanism, or if each similarly acting compound is present at a level well below its threshold, an additive effect would not be expected. When each component in a mixture is present at a level well below its respective threshold, it is also questionable whether synergistic or antagonistic interactions would be anticipated. In these cases, application of the additivity assumption and/or methods to account for synergistic and antagonistic interactions would not portray risk accurately.

Several steps are necessary to evaluate the validity of the hypothesis that adverse effects are unlikely when the components of a mixture are present at levels well below their respective thresholds. The first step is to establish the threshold for the endpoint of concern for each mixture component. Although it is rarely possible to establish the precise threshold, the threshold for observable adverse effects should lie somewhere between the NOAEL and the LOAEL. The second step is to assess the toxicity of the mixture using the same experimental protocol used to assess the toxicity of the individual components of the mixture. Finally, the relationship between the development of adverse effects following exposure to the mixture and the dose of the mixture components relative to their respective NOAELs and LOAELs can be established. If adverse effects do not occur when the dose of each component is well below its respective threshold and the threshold lies above the NOAEL, then adverse effects would not be anticipated when each component of the mixture is present at levels well below their respective NOAELs. Conversely, a range of effects would be anticipated when each component of the mixture is present at a level near, equal to, or greater than its individual threshold.

These kinds of scenarios have begun to be tested. Recently, several studies were conducted to examine the relationship between the development of adverse systemic effects and the dose of the mixture components relative to their NOAELs and LOAELs (Groten *et al.,* 1994; Jonker *et al.,* 1990, 1993a,b, 1994). In the first of this series of studies (Jonker *et al.,* 1990), a mixture of eight arbitrarily chosen compounds (sodium metabisulphate, mirex, loperamide, metaldehyde, di-*n*-octyltin chloride, stannous chloride, lysinoalanine, and potassium nitrate) was administered to rats for four weeks. When the dose of the constituent components was $\frac{1}{3}$ to $\frac{1}{10}$ of their respective NOAELs, no adverse effects were noted. When the constituent compounds were present at a concentration that approximated their respective NOAELs, some effects were occasionally noted, but they tended to be of minor toxicological significance. In contrast, when the constituents were present at their LOAEL values, a range of responses was observed. Several effects including growth retardation and liver damage were more severe than seen with the individual compounds at the same dose levels. Other effects including changes in the weight and morphology of the thymus were less severe in this study than after exposure to di-*n*-octyltin chloride alone. Some effects such as necrosis of the pancreatic acinar cells and hypertrophy of the zona glomerulosa of the adrenals were observed when the compounds were administered individually at doses equivalent to the LOAEL, but were not observed at all when administered at the same dose levels in the mixture. In addition, a number of effects such as reduced numbers of corpora lutea and increased numbers of multinucleated giant cells in the epididymides were observed after exposure to the mixture that had either not been seen when the compounds were administered individually or had only been seen at doses much higher than the LOAEL.

A subsequent study examined the toxicity of a mixture of nine arbitrarily chosen compounds administered to rats for 4 weeks (Groten *et al.,* 1994). Seven of the compounds were administered in the diet and included aspirin, cadmium chloride, stannous chloride, loperamide, spermine, butylated hydroxyanisole, and di(2-ethylhexyl)phthalate. In addition, formaldehyde and dichloromethane were administered by inhalation for 6 hr per day, 5 days a week. Preliminary results of this study indicate that when the dose of the nine constituent components was $\frac{1}{3}$ of their respective NOAELs, no adverse effects occurred. When the dose

of the constituent components was equal to their respective NOAELs, a few treatment-related effects occurred including hyperplasia of the nose, decreased kidney weight and alkaline phosphatase levels, and increased liver weight. However, when the dose of the compounds was equal to their respective LOAELs, a variety of effects occurred ranging from antagonism to additivity to synergism depending on the endpoint.

The chemicals used in the studies discussed above were arbitrarily chosen with respect to target organ and mechanism of action. Two subsequent studies were conducted to examine the effects of a mixture containing compounds known to act on the same target organ, but differing in their mechanisms of action. Four nephrotoxic compounds, mercuric chloride, potassium dichromate, *d*-limonene, and hexachloro-1,3-butadiene, were administered to rats in a single-dose study with sacrifice 24 hr later (Jonker *et al.*, 1993a) or over a 4-week period (Jonker *et al.*, 1993b). In these studies, there were either no effects when the dose of the constituent components was $\frac{1}{4}$ of their respective NOAELs or minimal effects when the doses were equal to the individual NOAELs. However, subacute (4-week) exposure to a mixture containing the compounds at a concentration equivalent to their respective LOAELs produced renal toxicity in male rats that was more severe than that observed after administration of any of the compounds individually. Acute (24-hr) exposure to this same mixture resulted in a range of effects from antagonism to additivity to synergism depending on the endpoint.

Although each of the compounds used in the studies conducted by Jonker *et al.* (1993a,b) were nephrotoxic, they exerted this effect via different mechanisms. To more fully test the validity of the additivity assumption, Jonker *et al.* (1994) conducted a 4-week study in rats of the toxicity of four compounds (trichloroethylene, tetrachloroethylene, HCBD, and 1,1,2-trichloro-3,3,3-trifluoropropene) thought to exert their effect via the same toxicological mechanism (initial conjugation with glutathione in the liver, followed by transport of the conjugate to the kidney and formation of reactive metabolites in proximal tubular epithelial cells). Preliminary results indicate that combined exposure to the four compounds at their respective NOAELs produced a slight increase in kidney weight, relative to controls. However, combined exposure to the four compounds at a dose equivalent to one-half of their individual LOAELs resulted in clear nephrotoxicity, as measured by alterations in multiple endpoints. This study did not include doses of mixture components that were less than their respective NOAELs.

The results of these studies point out the importance of defining "low dose" with respect to the threshold for each mixture component. In addition, these results are consistent with the hypothesis that adverse systemic effects are unlikely if the components in a mixture are present at levels well below their individual NOAELs, and therefore well below their thresholds. However, some adverse effects, albeit minor, can occur even when the mixture components are present at a level equivalent to their respective NOAELs, regardless of the mechanism by which the components exert their toxic effect. Furthermore, a range of interactive effects is possible when coexposure occurs to mixture components present at a level equivalent to their individual LOAELs.

The National Toxicology Program (NTP) and others have recently conducted several studies of complex mixtures, but did not conduct studies of the individual components of the mixture. Since they were not conducted to address the question of low level exposure within the framework outlined above, it is not possible to examine directly the validity of the hypothesis that adverse effects will not occur if the components of a mixture are present at levels below their individual thresholds. However, it is possible to use the NTP mixture studies to examine the hypothesis indirectly by selecting other benchmarks of toxicity for the individual components of the mixtures. Appropriate benchmarks for comparison are the NOAEL and LOAEL for a particular endpoint. In the absence of these data, other benchmarks are the NOAEL and LOAEL of the "critical study" that the U.S. EPA used to establish the reference dose (RfD) for the particular chemical of interest. Although the design of the "critical study" used to derive the RfD is not necessarily comparable to the design of the studies conducted by the NTP, presumably the NOAEL of the "critical study" represents the lowest NOAEL known.

For the purposes of further testing the hypothesis that adverse effects are unlikely when the components of a mixture are at levels below their individual thresholds, the EPA's Integrated Risk Information System (IRIS) was searched to find the RfD and the corresponding NOAEL and LOAEL for each of the chemicals in the mixtures tested by the NTP. These values were used to determine the likelihood that the thresholds of the components of the mixtures tested by the NTP were exceeded. The toxicity data obtained from the NTP studies and the benchmarks for the individual components of the tested mixtures are discussed below.

In the first series of studies conducted by the NTP, the toxicity of a mixture of 25 chemicals that are often found in contaminated groundwater near hazardous waste sites (Yang *et al.*, 1989) was examined. Chapin *et al.* (1989) prepared 1, 5, and 10% dilutions of the mixture in drinking water, and investigated the effects of these mixtures on spermatogenesis in $B6C3F_1$ mice after 90 days of exposure. There were no effects on mean body weight, no histological effects in the liver, kidney, testis, epididymis, or seminal vesicles, or on the absolute weights of these organs. Epididymal sperm number and testicular spermatid count were not af-

fected by treatment. The only effect noted was an increase in relative kidney weight in the high-dose group (10% solution).

The potential renal and hepatic toxicity of the same groundwater mixture, as well as the effect of exposure to the mixture on carbon tetrachloride (CCl_4) toxicity, was investigated by Simmons *et al.* (1994). Fifteen groups of F344 male rats received one of the following three drinking water treatments for 14 days: (1) deionized water *ad libitum,* (2) 10% drinking water solution of the groundwater mixture *ad libitum,* or (3) restricted deionized water. At the end of the 14-day treatment period, the rats were gavaged with 0, 0.0375, 0.05, 0.075, or 0.15 ml CCl_4/kg bw in corn oil. The restricted water control was added because reduced food and water consumption were noted in an initial study of the 10% drinking water solution. Rats receiving the 10% solution exhibited significant reductions in food and water consumption, and a significant increase in relative kidney weight. However, the water restricted group also exhibited a significant increase in relative kidney weight, suggesting that the increased kidney weight observed in the group that received the 10% solution may be due to reduced water and food intake. In addition, both the water-restricted and 10% solution groups exhibited small, but statistically significant, changes in several serum parameters; the authors questioned the biological significance of these changes. There was no evidence of hepatic toxicity in the three water treatment groups in the absence of CCl_4 treatment, and all three groups exhibited a dose-related increase in hepatotoxicity following CCl_4 exposure. However, the response of the groups receiving the 10% solution was similar to that of the water restricted groups, suggesting that most of the CCl_4-induced hepatotoxicity was due to restricted water and food intake.

A comparison of the concentration of each chemical in the NTP mixture (at the 10% dilution level) with the NOAEL or LOAEL of the critical study used to derive the RfD, as well as the RfD, was then made (Table 10). The RfDs of 2 of the 17 chemicals for which an RfD was available, arsenic and cadmium, were based on human data, and therefore the NOAELs should not be compared to the rodent data of Chapin *et al.* (1989) and Simmons *et al.* (1994). The RfDs of the remaining 15 chemicals were based on rodent data, and in all cases the doses administered in the Chapin *et al.* (1989) and Simmons *et al.* (1994) studies were well below the NOAEL of the critical study used to define the RfD. The results of this comparison are consistent with the hypothesis that adverse effects will not result from exposure to a mixture of chemicals provided that the concentration of each component in the mixture is well below its individual threshold.

Germolec *et al.* (1989) and Hong *et al.* (1992) investigated the potential immunotoxic and myelotoxic effects of this same drinking water mixture. Germolec *et al.* (1989) exposed female $B6C3F_1$ mice to a 0.2, 2, or 20% drinking water solution for 14 days, and to a 1, 5, or 10% solution for 90 days. In the 14-day study, the high-dose animals (20% solution) exhibited signs of immunotoxicity, including suppression of granulocyte–macrophage colony formation, suppression of antigen-induced antibody-forming cells, and resistance to challenge with an infectious agent. In the 90-day study, there was evidence of immunotoxicity in the mid- (5% solution) and high-dose (10% solution) groups. In addition, all treated groups showed signs of mild microcytic anemia. Similar results were reported by Hong *et al.* (1992). These investigators exposed female $B6C3F_1$ mice to a 1, 5, or 10% dilution of the mixture in drinking water for 2.5 to 31.5 weeks. There was evidence of immunotoxicity and myelotoxicity (suppression of granulocyte–macrophage progenitor cells and erythroid progenitors) in the mid- (5% solution) and high-dose (10% solution) groups after 15.5 weeks of exposure.

Although the results of these studies appear to refute the hypothesis that adverse effects are unlikely when the components in a chemical mixture are at levels below their individual thresholds, it is important to note that the endpoints assessed in the studies are not always assessed in the toxicity studies used to derive a RfD. Second, a RfD has not been determined for several components of the mixture known to cause immunotoxicity and myelotoxicity including benzene and aroclor (Table 11), so a comparison of threshold levels with the actual dose level used in the studies is not possible.

A second series of studies conducted by the NTP examined the toxicity of two drinking water mixtures of pesticides and fertilizers. One mixture simulated contaminants found in California and included the pesticides aldicarb, atrazine, dibromochloropropane, 1,2-dichloropropane, ethylene dibromide, and simazine, and the fertilizer ammonium nitrate. The other mixture simulated contaminants found in Iowa and included the pesticides alachlor, atrazine, cyanazine, metolachlor, and metribuzin and the fertilizer ammonium nitrate. Heindel *et al.* (1994) conducted two studies, a continuous breeding reproductive toxicity study with Swiss CD-1 mice and a developmental toxicity study with Sprague–Dawley rats. The animals were administered the California or Iowa mixtures in drinking water at three dose levels, 1X, 10X, and 100X, where 1X was the median concentration of each pesticide component as determined in groundwater surveys in the two states. In the reproductive toxicity study, neither mixture caused any clinical signs of toxicity, changes in food or water consumption, or body weight in either F_0 or F_1 mice at any dose level. There were no treatment related effects on fertility, measures of spermatogenesis, epididymal sperm concentration, percentage motile sperm, percentage abnormal sperm, or testicular or epididymal histology. In the developmental toxicity study, the pregnant dams were exposed to the mixtures

TABLE 10
Comparison of the Concentrations of the 25 Groundwater Contaminants in the Chemical Mixture with Their NOAELS, LOAELS, and Reference Doses

Component	mg/kg/day			
	10% Solution[a]	NOAEL[b]	LOAEL[c]	RfD[d]
Acetone	4.36	100.0	500.0	0.1
Aroclor 1260	ND	ND	ND	ND
Arsenic trioxide[e]	0.94	0.0008	0.014	0.0003
Benzene	1.25	ND	ND	ND
Cadmium acetate hydrate[e]	5.06	0.005	ND	0.005
Carbon tetrachloride	0.034	0.71	7.0	0.0007
Chlorobenzene	0.01	19.0	54.5	0.02
Chloroform	0.64	ND	12.9	0.01
Chromium chloride hexahydrate[e]	3.57	1468.0	ND	1.0
1,1-Dichloroethane	0.14	ND	ND	ND
1,2-Dichloroethane	3.47	ND	ND	ND
1,1-Dichloroethylene	0.045	ND	9.0	0.009
1,2-*trans*-dichloroethylene	0.26	17.0	175.0	0.02
Di(2-ethylhexyl)phthalate	ND	ND	19.0	0.02
Ethylbenzene	0.035	97.1	291.0	0.1
Lead acetate trihydrate[e]	6.93	ND	ND	ND
Mercuric chloride[e]	0.045	ND	ND	ND
Methylene chloride	3.4	5.85	52.6	0.06
Nickel acetate tetrahydrate[e]	0.62	5.0	50.0	0.02
Phenol	2.88	60.0	120.0	0.6
Tetrachloroethylene	0.30	14.0	71.0	0.01
Toluene	0.72	223.0	446.0	0.2
1,1,1-Trichloroethane	0.21	ND	ND	ND
Trichloroethylene	0.73	ND	ND	ND
Xylenes	0.145	179.0	357.0	2.0

[a] Values from Chapin *et al.* (1989).
[b] NOAEL of the study used to derive the reference dose (IRIS, 1994).
[c] LOAEL of the study used to derive the reference dose (IRIS, 1994).
[d] Reference dose (IRIS, 1994).
[e] The concentrations shown are for the metal ion, not the salt.

during gestation days 6–20. Neither mixture caused maternal or developmental toxicity.

To compare the dose levels of the individual components in each mixture with an estimate of their individual thresholds, the IRIS database was searched to determine whether a RfD had been established for each of the chemicals. RfDs were available for 4 of the 8 pesticides present in the California mixture, and for 4 of the 5 pesticides present in the Iowa mixture; a RfD was also available for the fertilizer component, ammonium nitrate (Table 11). It should be noted that reproductive and/or developmental toxicity data were available for these compounds, but in no case were these endpoints determined to be the "critical" endpoint. Therefore, the critical study used to define the RfD would presumably be below the threshold for reproductive and developmental effects. The dose levels of the four pesticides in the 100X Iowa mixture were well below the respective NOAELs of the study used to derive the RfD (Table 11). In the California mixture, the dose levels of atrazine, simazine, and aldicarb sulfone were well below the respective NOAELs used to derive the RfDs (Table 11).

The RfDs for aldicarb and ammonium-nitrate are based on human data, and therefore should not be compared to the rodent data of Heindel *et al.* (1994). However, there are rodent reproductive and developmental toxicity data available for aldicarb that provide an estimate of the thresholds for comparison. A rat two-generation reproductive toxicity study of aldicarb established a NOAEL of 0.4 mg/kg/day (IRIS, 1994). Although different species were used in the two studies, the lack of adverse effects in the continuous breeding study of the mixture suggests that the levels of aldicarb in the California mixture (0.061 mg/kg/day in the 100X dose level) were well below the effect threshold. A rat dietary developmental toxicity study established a NOAEL of 1 mg/kg/day, and a rat gavage developmental toxicity study established a NOAEL of 0.125 mg/kg/day (IRIS, 1994). Thus, the value of 0.042 mg/kg/day in the 100X group was well below the threshold for developmental toxicity.

No reproductive toxicity studies have been conducted for nitrate, but a rat 3-generation reproductive toxicity study of sodium nitrite in drinking water established a NOAEL of 20 mg nitrite-nitrogen/kg/day. The EPA

TABLE 11

Comparison of the Concentrations of the Pesticide/Fertilizer Components in the California and Iowa Mixtures with Their NOAELS, LOAELS, and Reference Doses

Component	mg/kg/day				
	100X[a]	100X[b]	NOAEL[c]	LOAEL[d]	RfD[e]
California mixture					
Aldicarb	0.061	0.042	0.01	0.025	0.001
Aldicarb sulfone	0.061	0.041	0.11	0.58	0.001
Aldicarb sulfoxide	0.061	0.041	ND	ND	ND
Ammonium nitrate[f]	35.7	25.0	1.6	1.8–3.2	1.6
Atrazine	0.01	0.0068	3.5	25.0	0.035
1,2-Dibromo-3-chloropropane	0.0002	0.0001	ND	ND	ND
1,2-Dichloropropane	0.092	0.063	ND	ND	ND
Ethylene dibromide	0.018	0.011	ND	ND	ND
Simazine	0.006	0.004	0.52	5.3	0.005
Iowa mixture					
Alachlor	0.017	0.012	1.0	3.0	0.01
Ammonium nitrate[f]	31.0	23.3	1.6	1.8–3.2	1.6
Atrazine	0.0095	0.006	3.5	25.0	0.035
Cyanazine	0.0076	0.005	ND	ND	ND
Metolachlor	0.0076	0.005	15.0	150.0	0.15
Metribuzin	0.012	0.007	2.5	37.5	0.025

[a] These values reflect the estimated daily intake for F_0 male mice in the continuous breeding study (Heindel *et al.*, 1994). Daily intake for F_0 female, and F_1 males and females were within ± 10%.
[b] These values reflect the estimated intake for pregnant dams in the developmental toxicity study (Heindel *et al.*, 1994).
[c] The NOAEL from the study used to derive the reference dose (IRIS, 1994).
[d] The LOAEL from the study used to derive the reference dose (IRIS, 1994).
[e] Reference dose (IRIS, 1994).
[f] Expressed as mg nitrate-nitrogen/kg/day.

assumed that a maximum of 10% of nitrate is converted to nitrite and calculated a NOAEL of 200 mg nitrate-nitrogen/kg/day (IRIS, 1994). It is therefore likely that the levels of 36 mg nitrate-nitrogen/kg/day present in the 100X California mixture and the 31 mg nitrate-nitrogen/kg/day present in the 100X Iowa mixture were well below the threshold for reproductive effects in rodents. A rat gavage developmental toxicity study of sodium nitrate and potassium nitrate established a NOAEL of 41 mg nitrate-nitrogen/kg/day. Therefore, the levels of 25 mg nitrate-nitrogen/kg/day in the 100X California mixture, and 23.3 mg nitrate-nitrogen/kg/day in the 100X Iowa mixture were below the threshold. The results of these comparisons are consistent with the hypothesis that adverse effects will not occur if the components of the mixture are present at levels below their respective thresholds.

In contrast to the situation with noncancer endpoints, it is generally assumed that a threshold does not exist for most genotoxic carcinogens. Therefore, defining "low dose" in reference to a threshold is not possible. Many studies of the potential interaction among carcinogenic components of mixtures have been conducted, but the vast majority of these studies employed doses of the mixture components known to increase the tumor incidence. Only a few studies of chemical mixtures have been conducted that have addressed the issue of low level exposure by examining the carcinogenic potential of mixtures in which the individual components are at levels that do not result in tumor formation when administered alone. For example, Ito *et al.* (1991) administered five heterocyclic amines to rats in their diet for 6 weeks at doses $\frac{1}{5}$ and $\frac{1}{25}$th of the dose known to result in liver tumor formation for each individual compound. There was a slight increase of glutathione *S*-transferase placental form (GST-P) positive foci in the livers of rats exposed to the mixtures compared to rats exposed to the individual compounds. The authors concluded that the results were consistent with additive effects or slight synergism.

In a subsequent study, Hasegawa *et al.* (1994a) administered 10 heterocyclic amines, individually at doses $\frac{1}{10}$ of that known to be carcinogenic or in combination at $\frac{1}{10}$ and $\frac{1}{100}$ the carcinogenic dose, to rats in their diet for 6 weeks. A significant increase in the number of GST-P positive foci was noted in rats exposed to the $\frac{1}{10}$ dose of the mixture, which was consistent with a synergistic effect. The number of GST-P positive foci for the rats exposed to the $\frac{1}{100}$ dose level did not differ significantly from control. Similarly, Hasegawa *et al.* (1994b) observed a synergistic effect for intestinal tumor development in rats exposed to a low dose ($\frac{1}{5}$ or $\frac{1}{25}$ of the carcinogenic dose) of five heterocyclic amines.

Warshawsky *et al.* (1993) reported that application

of a 0.1% dose of a mixture of five polycyclic aromatic hydrocarbons (anthracene, chrysene, pyrene, fluoranthene, and phenanthrene) to the backs of mice for 104 weeks resulted in a tumor incidence of 23%. Coexposure of mice to the same complex mixture and to 0.001% benzo(a)pyrene (BaP), a dose that produced no tumors when this compound was applied singly to mice, resulted in a 47% tumor incidence after 104 weeks. If the response was additive, one would have expected the tumor incidence upon coexposure to be equivalent to the response seen upon exposure to the mixture without BaP, indicating that the carcinogenic response at low doses of BaP under these conditions was synergistic.

Takayama *et al.* (1989) conducted a long-term study of a mixture of 40 carcinogenic compounds that target different organs, including the liver, intestine, thyroid, urinary bladder, and skin. The dose of each component of the mixture corresponded to $\frac{1}{50}$th of the TD50 (dose that produced tumors in 50% of the exposed animals). At the end of the 2-year exposure period, there was an increase in the incidence of follicular cell thyroid tumors and neoplastic liver nodules in the exposed rats. However, the authors could not determine whether the response was additive or synergistic.

DISCUSSION AND CONCLUSIONS

There has been considerable effort invested in developing methodologies for conducting risk assessments of chemical mixtures. Because of the differences in the complexity of chemical mixtures found in the environment, the diverse components of chemical mixtures, our inconsistent understanding of the mechanism by which an interaction may occur among mixture components, and the varying availability of data, it is becoming apparent that no one approach will suffice for conducting risk assessments of chemical mixtures. As a result, many of the methodologies have been developed in response to specific risk assessment problems, and the most successful have been those that incorporate mechanistic data where available. For example, the TEF approach was developed because of a need to conduct risk assessments of chlorinated dioxins and chlorinated dibenzofurans. This methodology was therefore designed as an interim risk assessment approach for structurally similar compounds, which operate on the same target tissues by similar mechanisms. The TEF approach has been a valuable "interim" tool to assess the toxicity of mixtures of several important classes of compounds, including PCDDs and PCDFs (NATO/CCMS, 1988; Ahlborg, 1989; Ahlborg *et al.*, 1992; Bellin and Barnes, 1989; Kutz *et al.*, 1990; Safe, 1990, 1993), PCBs (Ahlborg *et al.*, 1994; Barnes *et al.*, 1991; U.S. EPA, 1987, 1991; Safe, 1990, 1993) and polycyclic aromatic hydrocarbons (Nisbet and LaGoy, 1992; U.S. EPA, 1993). However, the use of this methodology for other chemical classes has been limited for several reasons, including a lack of clear understanding of the mechanism of action and the lack of a clear correlation among *in vivo* and *in vitro* assays. Other methodologies such as the pharmacodynamic model proposed by Clevenger *et al.* (1991) have been developed to incorporate specific mechanistic data. This is a valuable methodology for the specific biological systems that it was designed for, but different methodologies will be required for different biological systems.

Other efforts have been directed toward modifying the component-based method for mixtures risk assessment proposed by the U.S. EPA (1986), which assumes dose additivity for systemic toxicants and response additivity for carcinogens. Several modifications have been proposed to allow for the incorporation of data on interactions. For example, Mumtaz and Durkin (1992) proposed a weight-of-evidence approach to account for both synergistic and antagonistic interactions in the derivation of the hazard index. The NRC (1989) recommended that an additional uncertainty factor be used in the derivation of the hazard index when necessary to account for synergistic interactions. A basic premise of these modifications is that the additivity assumption is not always valid and that there is a need to account for interactions. This is based on studies of the interactions among binary mixture components which often deviate from additivity. However, caution must be exercised when incorporating data on interactions of binary pairs in risk assessments. First, most interaction data are derived from studies using a single high dose of one or both agents. Therefore, the observed interactions are specific for the doses studied, and interactions at those dose levels should not be assumed to predict effects at other doses. Second, interaction data from studies of binary pairs may not be predictive of the interaction that would occur in a more complex mixture. Third, few studies have addressed the temporal nature of interactions, which is an important component in determining potential toxicity. Finally, the limited number of binary pairs that have been studied are not representative of the universe of possible binary pairs, since the components are usually selected because an interaction is expected, based on prior information. Thus, generalization of interaction data obtained from binary pairs, and incorporation of such data into a quantitative risk assessment of chemical mixtures containing more than two components has not only been difficult, but may be of questionable validity.

When developing risk assessment methodologies for chemical mixtures, it would be useful to determine the conditions under which the additivity assumption is valid, and conditions under which synergistic and/or antagonistic interactions are likely to occur. The U.S. EPA (1986) suggested that additivity is likely when mixture components are present at low doses and when

the components induce the same toxic effect via the same mechanism of action. However, the definition of "low dose" was vague.

There are two sets of studies that are particularly informative in this regard: the TNO studies and the NTP studies. Studies of chemical mixtures conducted at the TNO Nutrition and Food Research Institute in the Netherlands (e.g., Groten *et al.*, 1994; Jonker *et al.*, 1990, 1993a,b, 1994) underscore the importance of defining "low" levels in reference to the threshold of the individual components of the mixture. Studies were conducted both with mixtures of components that act via a common mechanism and with mixtures of components that do not act via the same mechanism (and therefore do not meet the criteria for the additivity assumption). These studies neither support nor refute the additivity assumption when the mixture components are present at levels well below the NOAEL for the individual components. However, they do indicate that there was no discernible toxic response until the dose levels of the individual components approached or exceeded their individual thresholds. Regardless of the mechanism of action of the components of the mixture, a variety of interactions were observed when the individual components were present at levels equivalent to their individual LOAELs and therefore above their individual thresholds.

The studies conducted under the auspices of the NTP on the toxicity of well-defined complex mixtures of drinking water contaminants (Chapin *et al.*, 1989; Heindel *et al.*, 1994; Simmons *et al.*, 1994) were not specifically designed to examine the relationship between the toxicity of the mixture and the dose levels of the individual components relative to their individual thresholds. However, these studies generally support the hypothesis that adverse effects are unlikely when the mixture components are present at levels well below their individual thresholds.

The results of the NTP and TNO studies have some interesting implications for risk assessment of chemical mixtures. For example, they suggest that as long as the dose levels of all of the individual components of a mixture are well below their individual thresholds, adverse noncarcinogenic effects are unlikely. These studies also suggest that adverse effects are possible when the mixture components are present at levels just below their respective thresholds (e.g., at their NOAELs), and that additive, synergistic, and antagonistic interactions may occur (even simultaneously for different toxic endpoints) when the mixture components are present at levels equal to or above their individual thresholds. These observations apply regardless of the mechanisms of action of the individual components.

Although the studies described above suggest that adverse systemic effects are unlikely to occur in experimental animals as long as the individual components of the mixture are present at levels well below their respective thresholds, it is prudent to anticipate exceptions to the rule. In addition, these results should be extrapolated cautiously to humans. First, humans may be more or less susceptible to the toxic effects seen in the animal studies. In addition, and perhaps more importantly, humans may be exposed to many more compounds than those used in the animal studies including alcohol, therapeutic drugs, and environmental chemicals. These coexposures may increase or decrease the toxicity of mixtures to humans.

Clearly, more research is needed to evaluate the hazards and risks from exposures to mixtures of chemicals. Several animal studies demonstrate the potential for synergistic carcinogenic effects upon coexposure to very low doses of mixture components. Methodologies need to be developed to account for potential interactions. For noncancer endpoints, attention should be directed toward investigations of the relationship between toxicity of a mixture and the levels of the individual components relative to their individual NOAELs. In particular, mixtures containing compounds that are thought to act via a common mechanism should be examined; indeed, the question of what constitutes a "common mechanism" is still a thorny issue. Questions like these need to be addressed before progress can be made in improving the methodologies for risk assessment of chemical mixtures.

ACKNOWLEDGMENTS

The authors thank the members of the ILSI North America Residue Technical Committee for their interest, helpful comments, and financial support of the project. The secretarial support of Deborah Wilson and Andrea Gasper is greatly appreciated.

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