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PERSPECTIVE

Chemical Mixtures: An Unsolvable Riddle?

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ABSTRACT

It is difficult to overstate the complexity of assessing risks from chemical mixtures. For every valid reason to assess risks from mixtures, there appears an equally valid question as to whether it is possible to do so in a scientifically rigorous and relevant manner. Because so few data exist for mixtures, current mixture assessment methods must rely on untested assumptions and simplifications. That the accuracy of risk estimates improve with the number of chemicals assessed together as mixtures is a valid assumption *only* if assessment methods for mixtures are better than those based on individual chemicals. On the other hand, arbitrarily truncating a mixture assessment to make it manageable may lead to irrelevant risk estimates. Ideally, mixture assessments should be as broad as necessary to improve accuracy and reduce uncertainty over assessments that only use toxicity data for single chemicals. Further broadening the scope may be ill advised because of the tendency to increase rather than decrease uncertainty. Risk assessment methods that seek to be comprehensive at the expense of increased uncertainty can hardly be viewed as improvements. It would be prudent to verify that uncertainty can be reduced before burdening the risk assessment process with more complexity.

Key Words: chemical mixtures, drug interactions, chemical interactions, health risk assessment, data evaluation.

INTRODUCTION

Predicting the effects of chemical or drug mixtures has perplexed pharmacologists and toxicologists for decades. In pharmacology, one of the challenges has been to develop methods that predict whether combinations of medicines will interact adversely—either to increase toxicity or decrease efficacy. In toxicology, the primary challenge has been to develop methods for predicting the toxicity resulting from the

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combined exposure to large numbers of chemicals present in diverse environmental media ranging from ambient air and water to food, drugs, and consumer products. These challenges are formidable from biological, chemical, and statistical perspectives and present significant obstacles to those who would formulate methodologies for assessing risks from exposure to mixtures.

Perhaps the most intractable aspect of mixture toxicity assessment is the sheer magnitude of the task. Not only is the number of unique mixtures in the environment practically infinite, mixtures are constantly changing in composition and concentration due to transformation and transport processes within organisms and environmental media. Risk assessment methods cannot possibly account for the complexity of these ever-changing mixtures; hence, regulatory agencies have found it necessary to allow vast simplifications in mixtures risk assessment methods (Hertzberg and Teuschler 2002), including simplifications that allow risk assessors to use toxicity data for single chemicals rather than mixtures (ATSDR 2001a, 2001b; USEPA 1999, 2000).

The extensive use of simplifications in mixture risk assessment has received sharp criticism and led to legislative mandates that require an increased level of sophistication. For example, provisions in the 1996 U.S. Food Quality Protection Act require the USEPA to assess aggregate exposures from multiple pesticide uses and cumulative toxicity that occur by common mechanisms of toxicity. In many respects, the requirement to increase sophistication has led to replacing simplifications with mere assumptions, few of which have been scrutinized experimentally. Many assumptions about mixtures seem rational enough, but without clear experimental data to support them, it is impossible to know whether their adoption increases or decreases uncertainty in risk assessments. The overarching theme of this commentary is that both the simplifications and the assumptions made in mixture risk assessment should be judged, not on the basis of their necessity, but rather, by the degree to which they reduce uncertainty and lead to more scientifically defensible risk assessments.

ARBITRARILY DEFINING MIXTURES

One of the most significant challenges in mixture risk assessment is defining and delimiting the mixture of concern. Environmental chemical mixtures can be defined on the basis of the source of the chemicals that comprise them, the medium in which they are found, or the biological receptor(s) that may become exposed. This is not only an analytical chemistry problem; it is largely a conceptual issue fundamental to the purpose of the risk assessment. Regardless of the basis for the definition, implementing it usually requires assumptions and simplifications.

It is often assumed that a risk assessment will be more accurate (or more conservative and thus, protective) if it evaluates in some collective fashion all chemicals to which a receptor is exposed rather than only a subset of those chemicals. This may seem intuitively reasonable, but in practice, most mixture assessments are spatially and temporally constrained. Typically, the chemicals evaluated as a mixture are only those that enter the environment from a particular source or that might be encountered at a particular site, and include only the set of chemicals measured at a single point in time rather than the sequence of chemicals that actually exist over time. Presumably, these simplifications constrain the scope of the assessment exercise to

make it manageable and to limit the range of risk mitigation strategies to those for which a responsible party may be held liable.

However practical this situation may appear, it seems reasonable, nonetheless, to ask whether the most toxicologically significant mixture effects are likely to occur between the chemicals encountered in the same place or between combinations of chemicals encountered in different places. For pharmaceuticals, one might posit that the mixture of concern should be delimited to the suite of prescription or over-the-counter (OTC) medications typically taken in combination, based on market survey or prescribing frequency data. However, delimiting the mixture so narrowly would not account for important pharmacological interactions that may occur with foods and dietary supplements. For example, grapefruit juice has been shown to enhance the pharmacological effect of a number of drugs due to inhibition of gastrointestinal drug metabolism and consequent increased GI absorption (Kane and Lipsky 2000) and several interactions are suspected with St. John's Wort due to cytochrome P450 inhibition (Moore *et al.* 2000).

In similar fashion, environmental toxicology must ask whether it is sufficient to assess the milieu of chemicals present at a particular site or within a particular medium rather than to evaluate the entire suite of chemicals to which a potential receptor might be exposed. For example, risk assessments to support occupational safety and health decisions might focus on the chemicals used in manufacturing in a particular work environment, ignoring the possibility that the majority of a worker's chemical exposures might occur outside of work from food, drugs, consumer products, and chemicals used in residential and other non-occupational environments. Not only does delimiting the mixture temporally and spatially reduce the number of mixture components arbitrarily, this practice ignores the importance of sequence of exposure and past toxic insults in determining the toxicity of many chemical combinations. The USEPA (2000) has acknowledged these issues by defining chemical mixtures irrespective of the spatial or temporal characteristics of their components.

Thus, on one hand, it is important to include all toxicologically significant chemicals in the mixture assessment, but on the other hand, it is usually necessary to limit the assessment to a manageable number of mixture constituents. There is a critical need to balance these contravening goals, but currently, no broadly applicable scientific method exists for doing so. Ideally, the mixture assessment should be only as broad as necessary to improve accuracy and reduce uncertainty over an assessment that considers only the toxicity of individual chemicals. Further broadening the scope of mixtures assessment would be ill advised because of the tendency to increase rather than decrease uncertainty. Assuming that the accuracy of mixture risk assessments improves with the number of mixture components assessed is valid *only* if the methods for predicting combined toxicity are better than methods based on toxicity for individual chemicals alone. If the primary reason for assessing mixtures were merely to increase conservatism in the risk estimate rather than improve accuracy, there are much simpler ways of achieving that end than attempting to predict combined toxicity. For some particularly important risk assessment goals, such as protecting infant health, conservatism may not be synonymous with health protection (Borgert *et al.* 2003).

PREDICTING THE UNPREDICTABLE—INTERACTION, NON-INTERACTION, AND MODE OF ACTION

One of the primary reasons for assessing mixtures rather than simply adding risks for individual chemicals is to address the concern that risk estimates for single chemicals might grossly underestimate toxicity due to the potential for synergism between mixture components. Indeed, the specter of synergism has been used to raise concerns about a range of different chemical mixtures from prescription diet aids to environmental estrogens. Notoriety aside, it is important to consider objectively the likely public health and environmental consequences of synergistic interactions (Groten 2000). A synergistic interaction can be extremely useful and economically valuable when it confers therapeutic advantage or reduces the opportunity for acquired resistance in viruses, cancers, or among microbial or insect pests. For this reason, countless resources have been spent by the pharmaceutical and pesticide industries to develop useful synergistic combinations for medicine and agriculture. With a few exceptions, these efforts have been generally unsuccessful. Although many therapeutically advantageous drug combinations have been identified, this should not be taken as evidence of drug synergism; pharmacologic addition, toxicologic antagonism, and a reduced chance of tolerance or resistance developing in target organisms are more common means of conferring therapeutic advantage (Berenbaum 1988). Furthermore, it can be shown mathematically that interactions, including synergism, are more likely to occur in the mid-range of the dose-response curve than at either high or low extreme (Berenbaum 1989). Consequently, synergism should be easier to identify among pharmaceuticals, which are used in biologically active concentrations, than among environmental contaminants present at concentrations below the observable effect range. The fact that pharmacologists and toxicologists have found so few biologically significant synergistic interactions, despite great scientific, professional and financial motivation to do so, suggests that these interactions are probably also rare in the environment.

Regardless of the probability that synergistic interactions are causing significant environmental or clinical problems, a satisfactory method to evaluate synergism in risk assessments would be a welcome advancement. A methodology that can identify the chemical combinations most likely to be synergistic would limit the scope of a mixtures assessment to a manageable number of chemicals and simultaneously focus the assessment on the components of greatest concern. Such methods have been proposed (Durkin *et al.* 1995). However, due to the complexities discussed in this commentary, such elegant methods are much more easily conceived than implemented for most chemicals.

The first challenge for addressing synergism is to define it. Within the field of interaction pharmacology and toxicology, there has been considerable debate over the proper definitions of terms such as *synergism*, *antagonism*, *potentiation*, and *additivity*, and as a result, there appears to be widespread confusion over terminology outside this narrow field. The confusion is responsible, in part, for the limited amount of interaction data useful for risk assessment (Hertzberg and McDonnell 2002). Synergism can be defined broadly as a type of "interaction" in which chemicals produce more toxicity as a combination than would be predicted by their actions separately. Another way of stating this concept is that lower concentrations of chemicals are

required to produce a particular magnitude of effect when the chemicals are administered together than would be predicted from their actions separately. Antagonism is the converse; *i.e.*, less effect produced, or more chemical required, than predicted.

The word "predicted" is of critical importance in these definitions. It is important to appreciate that synergism cannot be inferred whenever a mixture of chemicals produces an effect greater than the same chemicals administered separately. Rather, the determination requires a comparison of the observed effect with the effect *predicted* based on the concentration-effect relationships of the individual components of the mixture. The *predicted* effect can be derived by applying either concentration addition or independence, both of which are widely accepted as valid models of non-interaction (Berenbaum 1981). These concepts have been reviewed extensively elsewhere (Berenbaum 1989; Greco *et al.* 1995; Cassee *et al.* 1998; USEPA 2000; Borgert *et al.* 2001; Tallarida 2001). For risk assessment, the critical issue is that interactions—synergism and antagonism—cannot be directly tested; rather, these interactions are inferred from experimental results that deviate from a model of non-interaction based on the concentration-effect characteristics of the individual mixture components. Thus, with regard to predicting synergism (or antagonism), toxicologists and risk assessors face the conundrum of predicting dose-response phenomena that are, by definition, not readily predicted by any simple model.

One way to avoid the need to predict interactions is to assume that in mixtures containing many chemicals, synergism and antagonism will essentially cancel one another. Predicting the toxicity of the mixture is then a matter of predicting which chemicals will be non-interactive according to independence (response addition) and which will be non-interactive according to concentration addition (dose addition). In other words, rather than attempt to predict interactions, the focus is on how to add the toxicity of chemicals in a mixture. Concentration addition (or dose addition) is based on the concept that a single chemical does not interact with itself, and thus, multiple doses of one chemical are non-interactive (Loewe and Muischnek 1926). Predicting that two 325 mg aspirin tablets will produce the same analgesic effect as a single 650 mg tablet is a simple example of dose addition. Current risk assessment methodologies typically extend dose addition to groups of chemicals with similar modes of action based on the assumption that one chemical can be replaced by an equi-effective concentration of any similarly acting chemical. Continuing with the analogy, the analgesic effect of 325 mg aspirin tablet and 200 mg ibuprofen could be predicted by summing the ratio of dose to relative analgesic potency for each drug. In risk assessment, this has become known as the toxic equivalency, or TEQ approach. The TEQ approach was developed for true chemical congeners that share pharmacokinetic and pharmacodynamic behavior, molecular targets, and have parallel dose-response curves, but is probably inappropriate for chemicals that deviate significantly from these requirements (Safe 1998). The TEQ approach was initially applied to assess risks posed by mixtures of dioxins and dibenzofurans (Safe 1990), but recent data and conceptual concerns call into question its applicability for these (Toyoshiba *et al.* 2004) and other groups of chemicals (Safe 1998; Borgert *et al.* 2003). The hazard index calculation used in CERCLA-style risk assessments is another example of concentration addition, wherein the mixture is assessed by summing the ratio of received dose to reference dose (RfD) for each component.

In contrast to concentration (dose) addition, independent action (also called response addition) is based on a model of probabilistic independence and assumes that the toxicity of chemical A is unaffected by the presence of chemical B in a mixture (Bliss 1939). Independence is the non-interaction model recommended for mixtures of chemicals that act by different modes of action (ATSDR 2001a, 2001b; USEPA 2000). Cancer risk calculations are an example of response addition, wherein the overall risk for cancer from a mixture is calculated from the risks posed by each individual component. Current risk assessment guidance for chemical mixtures also recommends the use of response addition for chemicals that act dissimilarly in producing toxic endpoints other than cancer. Independence and concentration addition can be expected to give the same prediction only when applied to linear dose-response curves that intersect the origin of the dose-response plot. Under all other conditions, independence and concentration addition models are likely to yield different results (Greco *et al.* 1995). A special case of independent action, called effect summation, predicts mixture toxicity by summing the effects of the individual components. In some circumstances, effect summation can yield the paradoxical result that a chemical is synergistic with itself (Berenbaum 1989). For chemicals that exhibit a toxicity threshold, effect summation would predict a mixture effect of zero when all mixture constituents are present below the threshold concentration, whereas concentration addition could predict a supra-threshold response. To illustrate, consider a mixture of three nephrotoxic chemicals, each present at one-half its threshold concentration for producing tubular acidosis. Effect summation would predict a sub-threshold effect for the mixture (*i.e.*, $0 + 0 + 0 = 0$) whereas concentration addition would predict measurable tubular acidosis ($0.5 + 0.5 + 0.5 = 1.5$). Risk assessors should be aware that the important differences between various non-interaction models are often ignored in the published literature.

Predicting mixture toxicity for risk assessment has thus become an exercise in choosing between models of non-interaction based on the presumed mode of action of mixture components. This is an interesting and somewhat paradoxical development because an empirical test for interactions has often been used to differentiate chemicals that act by similar versus dissimilar mechanisms (Dawson and Poch 1997; Borgert *et al.* 2004a). From a practical perspective, it would seem that interaction studies are potentially much more informative about mechanisms than mechanistic studies are about interactions (Tallarida 2001; Borgert *et al.* 2004b). Although concentration addition has been verified at the molecular and cellular level for some chemicals with similar molecular targets (Silva *et al.* 2002) and for some nephrotoxicants, the general case remains to be established at the level of organisms or populations (Groten 2000).

Discerning the mode of action for all components of a mixture might appear to be more tractable than predicting synergism or antagonism, but it is a complex issue in practice. A mode of action can be viewed as a category of mechanisms that share particular key features or steps. Although several sets of criteria have been set forth for identifying the key mechanistic features that define a mode of action (ATSDR 2001a, 2001b; USEPA 1999, 2000, 2003; Mileson *et al.* 1998), the degree to which those key features must be understood in order to predict combined toxicity has not been established on the basis of data (Borgert *et al.* 2004b). Complexities that may need to be experimentally explored include interaction thresholds

(el-Masri *et al.* 1996a,b), causal relationships between various mechanistic steps, pharmacokinetic behavior (Haddad *et al.* 2000), mechanisms of interaction, and the dose-dependence of various toxicity mechanisms (Borgert *et al.* 2004b).

The mechanisms by which chemicals interact and the dose-dependence of those mechanisms may prove to be the most critical of all issues to address, particularly the dose-dependence of biological effects and interactions in the sub-threshold range for observable toxicity. The work of Hermanns and colleagues (see review in McCarty and McKay 1993) is perhaps the most innovative attempt at predicting the toxicity of complex mixtures containing components below their individual threshold concentrations for observable toxicity. Using standard aquatic toxicity models, these researchers showed that at sub-threshold concentrations, mixtures of organic chemicals fail to exhibit the toxicities of the individual components, but instead, conform to concentration addition for general narcosis. The narcotic potency of the mixture can be estimated quite accurately from the concentrations and the octanol-water partition coefficients of the mixture components.

Rather than attempting to predict interaction or non-interaction, it would seem that computing mixture toxicity from empirical interaction data would be a more direct means of estimating mixture toxicity and would reduce uncertainty in mixture risk assessments. Indeed, computational methods have been devised to predict the toxicity of complex mixtures based on pair-wise interaction data for mixture components (Haddad *et al.* 2000), and these methods have been validated for small sets of test mixtures (Haddad *et al.* 2001). A weight of evidence procedure (Mumtaz and Durkin 1992) that modifies the traditional hazard index calculation based on published interaction data is currently used to develop interaction profiles for various chemical mixtures (ATSDR 2001a,b). Unfortunately, published interaction data are lacking for the vast majority of drugs and environmental contaminants (Hertzberg and Teuschler 2002), and so the weight of evidence procedure is limited in its application. Moreover, many published interaction studies suffer serious methodological deficiencies that limit their use in risk assessment (Borgert *et al.* 2001). Some of the more common problems stem from an apparent misunderstanding about the nature of interaction and non-interaction (Berenbaum 1989) and misunderstanding of the statistical methods required for testing and interpreting interactions (USEPA 1990). The fundamental criteria for designing and interpreting interaction studies include the need to adequately assess dose response curves of the component chemicals individually and in combination, the need to test a specific no-interaction hypothesis using appropriate statistical tests, and the need to evaluate the interaction at relevant levels of biological organization (Borgert *et al.* 2001).

JUST TEST THE MIXTURE

One great advantage of predicting mixture toxicity from data on individual chemicals or combinations of a few chemicals is that theoretically, the data can be applied to many different mixtures containing different chemicals in different ratios and proportions. However, considering the difficulty of estimating mixture toxicity from data on individual components, their mechanisms of action, and data on their interactions, one might ask why not simply perform toxicity testing on the mixture of

concern, treating it as a unique single substance? This approach, sometimes called the "whole mixture approach," obviates any need to identify toxicity interactions produced by mixture components because these will be reflected in the toxicity of the mixture itself. The toxic effects and potency of the mixture can be assessed as is routinely done for single chemicals. This would simplify the determination of LOECs (lowest observed effect concentrations) and NOECs (no observed effect concentrations) if the data are more directly applicable to the mixture of concern and thus more readily interpretable than data on individual components or mixtures of only a few chemicals.

Of course, the whole mixture approach is not without significant limitations. In order to conduct toxicity tests, many mixtures would have to be extracted from the environmental medium in which they occur, and then concentrated (or diluted) to conduct toxicity tests. Because the identity, the concentration, and the relative proportions of constituents can affect mixture toxicity, any of these manipulations could introduce differences between the mixture tested and the mixture found in the environment. Such differences could reduce the relevance of the results. Finally, the sheer number of unique mixtures that exist in the environment precludes testing each, and indeed, it would be nearly impossible to completely identify and quantify every last component of even one mixture, let alone characterize the changes in mixture composition that occur with time.

For this latter reason, guidance documents for mixture risk assessment (USEPA 2000; ATSDR 2001a,b) recommend using data on a similar mixture as a surrogate for the mixture of concern. Indeed, the ability to conduct toxicity tests on a surrogate mixture and apply the data to many different environmental mixtures is appealing from both scientific and practical perspectives, but again, limitations and challenges abound. Foremost is the challenge of defining the level of similarity necessary to extrapolate toxicity data from one mixture to another. It seems reasonable that some degree of both toxicological and chemical similarity would be important for such extrapolations, but currently, there is no consensus on what chemical and toxicological features are essential. Nonetheless, extrapolating data from surrogate mixtures to environmental mixtures of concern is likely to be an important tool for mixtures risk assessment. Computerized methods that "lump" chemicals into groups based on physical chemical properties, structure activity relationships, and pharmacokinetic modeling may be applicable for some types of complex mixtures (Verhaar *et al.* 1997). For mixtures generally, it will be important not only to articulate clear guidelines for determining when two mixtures are sufficiently similar to justify using one as a surrogate for another, but also to formulate a method to verify that the guidelines are reliable.

CONCLUSIONS

In summary, it seems that for every valid reason to assess risks posed by chemical mixtures, there remains an equally valid question as to whether it is possible to do so in a scientifically rigorous and relevant manner. Until the scientific and technical challenges are overcome, it is incumbent on risk assessors to evaluate the uncertainties inherent in various approaches to mixture risk assessment and

to clearly communicate those uncertainties. Risk assessment methods that seek to be comprehensive at the expense of increased uncertainty can hardly be viewed as improvements. We might do better to verify that we can reduce uncertainty before burdening the risk assessment process with more complexity.

One way of reducing uncertainty might be to focus future research on identifying the mechanisms by which chemicals are most likely to interact in toxicologically significant ways, and on developing rapid assays to identify the chemicals that can participate in those mechanisms. Similar types of approaches have been explored for use in drug development (Hori 1997). For environmental risk assessment, it would seem most productive to focus on mechanisms of interaction that can occur at environmentally relevant concentrations, and to identify dose-dependent transitions in those mechanisms. Pharmacokinetic rather than pharmacodynamic mechanisms would seem to be more likely sources of toxicologically significant interactions, based on published literature (Krishnan and Brodeur 1991). Statistical optimization techniques may hold promise for determining the degree of mixture complexity at which various assessment methods contribute more uncertainty to the risk estimate than alternative methods for single chemicals. Ultimately, improving mixture risk estimates depends on developing clear hypotheses that allow us to test, refine, and validate the underlying assumptions.

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Short Communication

Can mode of action predict mixture toxicity for risk assessment?

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Abstract

Recent regulatory guidance for mixture risk assessments and for regulating pesticide chemicals recommends using information about the "mode" or "mechanism" of action of individual chemicals to predict dose response characteristics of mixtures. Dose addition is assumed for mixtures of chemicals that have similar mechanisms and response addition for those with dissimilar mechanisms. Three different sets of criteria have been formulated to guide the selection of an appropriate data set for characterizing a chemical's mode of action, but the sufficiency of those criteria to predict dose addition for a mixture has not been validated experimentally. Several examples from the pharmacological and toxicological literature challenge the premise that dose response characteristics of a mixture can be predicted from the modes of action of its components. Detoxification pathways may need to be understood before dose addition in the observable effect range can be extrapolated to mixture concentrations below the no observable effect levels of the mixture components. Because elucidating discreet mechanisms of action may be possible only for chemicals that exhibit a high degree of biological specificity and dose sensitivity, practical limitations on the approach must be defined. To reduce the large uncertainties inherent in the recommended approach, future research should be focused on defining the mechanistic features that predict dose additive toxicity in mixtures. A detailed characterization of pharmacodynamics, pharmacokinetics, and slope of dose response curves may be necessary to evaluate whether the toxicity of a mixture can be predicted by the mode of action of its component chemicals.

Keywords: Mixtures toxicity; Risk assessment; Mode of action

Introduction

Recent regulatory guidance for conducting mixture risk assessments and for regulating the cumulative risk posed by pesticide chemicals advocates using mechanistic information about individual chemicals to select models for predicting the dose response characteristics of a mixture (ATSDR, 2001a, 2001b; U.S.EPA, 1986, 1988, 1999, 2000a, 2000b, 2001). Specifically, those guidance documents recommend use of dose addition models to assess chemicals exhibiting similar mechanistic features and use of response addition (i.e., independence) models to assess chemicals exhibiting dissimilar mechanistic features. Such mechanism-based approaches can potentially be a great improvement over approaches that do not utilize mechanistic information to assess the toxicity of mixtures, such as the Hazard Index approach (U.S.EPA, 1989), which only considers whether similar target organs are affected.

Whether mechanism-based approaches will actually improve mixture risk assessments depends on the scientific validity and practical applicability of two underlying assumptions: that the mechanistic similarity between substances can be determined adequately, and, that in mixture, chemicals with similar or dissimilar mechanistic features display dose additive or response additive toxicity, respectively. This paper examines the scientific basis for those assumptions and identifies practical and theoretical limits for using mechanistic information to predict mixture toxicity. This paper also suggests future research that could guide the development and use of mechanistic data in mixture risk assessment.

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Models of non-interaction: response addition versus dose addition

Before examining the assumptions underlying mechanism-based approaches for assessing the toxicity of mixtures, it is helpful to understand the difference between independence (also known as response addition) and non-independence (also known as dose addition) models and how those models can impact dose response predictions for a mixture. Both models are "non-interaction" models, that is, they assume that chemicals are simply additive, and neither synergistic nor antagonistic, when combined in mixtures. For convenience, we refer to the toxicity produced when chemicals are combined in mixtures as "combined action."

The independence model of combined action is based on toxicological independence described by Bliss (1939). Independence, sometimes called "response addition," assumes that the toxicity of a mixture is the sum of the toxic effects of each constituent. For example, independence predicts that a mixture of chemicals will not exert an adverse effect when individual chemicals in that mixture are present below their individual No Observable Adverse Effect Level (NOAEL). According to U.S.EPA (2000a) and ATSDR (2001a, 2001b), independence should be used for mixtures of chemicals that produce the same toxic effect in the same target organ, but which do so via dissimilar mechanisms of action.

In contrast, dose addition, as described by Loewe and Muischnek (1926), is based on the assumption that non-interacting chemicals in a mixture behave as dilutions of one another and, therefore, may be related by potency factors (for a review, see Greco et al., 1995). The practical relevance of this for risk assessment is that dose addition predicts that a mixture of three chemicals, each present at a concentration one-half its toxic threshold, would produce a measurable toxic effect. According to U.S.EPA (2000a) and ATSDR (2001a, 2001b), dose addition should be used for chemicals that produce the same toxic effect in the same target organ via the same mechanism of action.

Regulatory toxicology has traditionally applied independence only to chemical carcinogens and dose addition to non-carcinogens, as in the Hazard Index approach. Although both models involve summing (either the component doses or their toxic effects), differences between models may produce large differences in the risks estimated for a particular mixture (U.S.EPA, 2000a) as illustrated in Fig. 1. The notable exception to this regulatory practice is the approach to dealing with the carcinogenicity of dioxin (TCDD)-like chemicals. Here, a dose-addition concept is used to calculate a single toxic equivalents (TEQ) value for all dioxin congeners (chlorinated dibenzo-*p*-dioxins and dibenzofurans) in a mixture based on their potencies relative to 2,3,7,8-TCDD, the designated reference compound. The procedure involves multiplying the toxic equivalency factor (TEF) (i.e., a relative potency value) for each congener in a mixture by its concentration and adding the products to derive the TEQ for the mixture. The cancer risk is then the product of the TEQ multiplied by the cancer slope factor for 2,3,7,8-TCDD, and this cancer risk is added together with cancer risks posed by all other carcinogenic chemicals at a site, a process that is consistent with independence (response addition). Thus, the practice for dioxins utilizes dose addition to obtain TEQs and response addition to combine the cancer risks posed by dioxin TEQs with those posed by other chemicals.

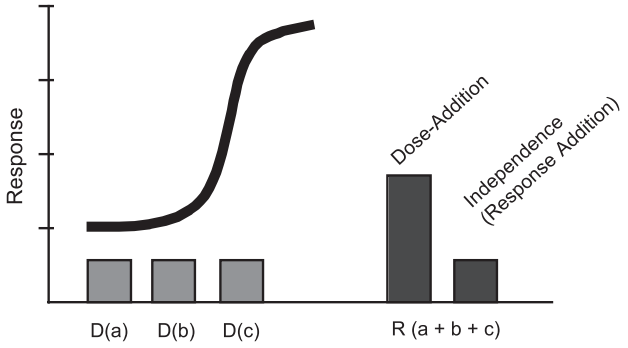

Fig. 1. A model dose-response curve is shown for an effect with a threshold. Below the curve, bars indicate doses D(a), D(b), and D(c) each of which is one-half the toxic threshold for chemicals a, b, and c, respectively. The calculated combined response R (a + b + c) would be greater or less than the threshold depending on whether a dose addition or independence model was used to predict the combined action of chemicals a, b, and c in a mixture.

"Mode" versus "mechanism" of action

Two different biological concepts—"mode of action" and "mechanism of action"—have been used to determine the extent to which chemicals exhibit similar mechanistic features, and therefore, to select the model of combined action for those chemicals in a mixture. Although the terms "mode" and "mechanism" are well defined, the toxicologic literature on mixtures and regulatory guidance documents for mixture assessments often fail to make clear distinctions between these terms. The distinction between "mode" and "mechanism," however, is critical to conducting a mixtures risk assessment. This is because choice of a model to predict the effects of chemical mixtures (i.e., a dose addition model versus a response addition model) can turn on whether mechanistic data for the chemical components of the mixture are described in terms of the mode or mechanism of action. Because of the importance of these concepts for choosing between dose addition and response addition models, it is important to understand the differences between these concepts and how common practice has blurred the distinction.[1]

[1] We make no conceptual distinction between the terms "mechanism of action" and "mechanism of toxicity," nor between the terms "mode of action" and "mode of toxicity." The latter terms in each pair simply specify drug or chemical actions that produce adverse effects. We thus use the terms interchangeably for this discussion, with the understanding that not all effects are adverse.

As traditionally used in pharmacology and toxicology, "mechanism of action" denotes the molecular sequence of events leading from the absorption of an effective dose of a chemical to the production of a specific biological response in the target organ (Butterworth et al., 1995; Dellarco and Wiltse, 1998; Schlosser and Bogdanffy, 1999; U.S.EPA, 2000b, 2001). Understanding a chemical's mechanism necessarily entails understanding the causal and temporal relationships between the steps leading to a particular effect, as well as the steps that lead to an effective dose of the chemical at the relevant biological target(s) of action. Therefore, to define a mechanism of action, experimental or clinical data would need to be sufficient to draw conclusions regarding the following for each effect of the chemical:

(1) metabolism and distribution of the chemical in the organism or population and subsequent modulating influence on the dose delivered to the molecular targets of action;
(2) molecular target(s) of action;
(3) biochemical pathway(s) affected by the chemical's action on the molecular target and resulting perturbations of those pathways;
(4) cellular and organ-level consequences of affecting the particular biochemical pathway(s);
(5) target organ(s) or tissue(s) in which the molecular target and biochemical affect occur;
(6) physiological response(s) to the biochemical and cellular effects;
(7) target organ response(s) to the biochemical, cellular, and physiological effects;
(8) the overall effect on the organism;
(9) for ecological effects, the overall effect on the population or ecosystem;
(10) causal and temporal relationships between the mechanistic steps;
(11) dose response parameters associated with each step.

In contrast, "mode of action" is a more general description of drug or chemical action (Dellarco and Wiltse, 1998; Schlosser and Bogdanffy, 1999; U.S.EPA, 2000b, 2001). Mode of action refers to the type of response produced in an exposed organism or to only the critical steps or features of the mechanism required for production of the particular biological response. For example, Rand et al. (1995) define mode of toxicity as "a common set of physiological and behavioral signs that characterize a type of adverse biological response." Schlosser and Bogdanffy (1999) define mode of action as a class or category of mechanisms that shares general features critical to the production of toxicity. Thus, the mode of action of a chemical is known if the full mechanism is known, but the reverse is not true. Overall, it is fair to conclude that the mode of action classification should consider some aspect of the critical biochemical pathway plus the resultant physiological and behavioral changes produced by alterations in that pathway by the toxic agent.

The distinctions between "mode" and "mechanism" are important for understanding and describing the actions of drugs and chemicals, but can become quite vague in practice because both mode and mechanism are often used to refer to a critical molecular event, that is, to a single important mechanistic step. For example, non-steroidal anti-inflammatory drugs (NSAIDs) are a class of chemicals related by a similar mode of action, inhibition of prostaglandin biosynthesis (Roberts and Morrow, 2001). Each drug within the class is differentiated by its mechanism, which can confer therapeutic advantages or disadvantages under specific conditions or in particular patients. However, because these drugs were developed to act on a specific mechanistic target, pharmacologists may refer to the mechanism of action of COX 2 inhibitors—a subset of NSAIDs—as simply inhibition of the type 2 isoform of the cyclooxygenase enzyme (COX 2). Alternatively, and confusingly, COX 2 inhibitors may be said to have a distinct mode of action from other NSAIDs because they inhibit a specific isoform of cyclooxygenase.

In toxicology, distinctions between "mode" and "mechanism" are also blurred by the way the discipline is organized and presented. For example, prominent toxicology textbooks are often organized according to various categories of chemical toxicity, yet no two textbooks use the same scheme. Some textbooks refer to these schemes as categories of mechanisms, others as categories of mode, and still others as categories of target organs and systems. Some clarity can be achieved, however, by careful reading of various texts. Gregus and Klaassen (2001) point out that several different approaches are actually required to categorize chemicals by their action, including physical chemistry, target organ, and mode of action. Timbrell (2000) suggests that two components must be considered to understand the mechanism of interaction of a foreign compound with the body: (1) the effect of the body on the compound, and (2) the effect of the compound on the body (synonymous with the terms "pharmacokinetics" and "pharmacodynamics," respectively). Timbrell asserts that the interaction of a chemical with macromolecules and the resulting physiological responses involve only the second component. Thus, grouping chemicals according to a categorization scheme is a useful didactic tool for introducing the broad array of chemical effects, but does not define mechanism or mode because these various categorization schemes address only one side of the equation needed to describe the toxic mechanisms of a chemical (i.e., only pharmacodynamics).

As is true for toxicology textbooks, US government agencies show little consistency with respect to the use of mechanistic concepts and terminology, and there are often inconsistencies even within a single agency. For example, EPA's proposed Cancer Risk Assessment Guidelines (U.S.EPA, 2001) make clear distinctions between the terms mode and mechanism of action according to traditional

Table 1
Use of "mode" or "mechanism" in regulatory guidance

Guidance document	Terminology	Use of mechanistic data	Definition or criteria
Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures. U.S.EPA, 2000a. pages 20–22, 28, 75–76.	mode or mechanism	To choose between dose additivity and response additivity models.	Chemicals are dose additive if 'chemical B is a functional clone of chemical A'. Dose additive chemicals have 'similar uptake, metabolism, distribution, elimination, and toxicologic properties', and there is a 'constant proportionality between effectiveness' such that their DRCs are 'congruently shaped', that is, 'parallel'.
Draft Final Guidelines for Carcinogen Risk Assessment. U.S.EPA, 2001. pages 1–5 to 1–15.	mode	Decide relevance of animal data; identify sensitive subpopulations; high to low dose extrapolation and predict threshold.	Mode of action is composed of key events and processes starting with interaction of an agent with a cell, through operational and anatomical changes, resulting in cancer formation. Mechanism of action implies a more detailed, molecular description of events than mode of action. To demonstrate mode, an understanding of the complete sequence of events at the molecular level (mechanism) is not expected; instead, use empirical observations at different levels of biological organization: biochemical, cellular, physiological, tissue, organ, system, and determine causal relationship between the events.
Dellarco and Wiltse, 1998. U.S. Environmental Protection Agency's revised guidelines for carcinogen risk assessment: incorporating mode of action data. Mutation Research 405: 273–277.	mode	Reduce uncertainty in carcinogen risk assessment; improve extrapolation of animal data to humans; predict thresholds.	Emphasizes the importance of understanding how environmental agents are changed through metabolism, the dose at the affected organ system, how an agent produces its adverse effect at high and low doses. "It should be noted that the term mode of action is deliberately chosen in these new guidelines in lieu of mechanism to indicate using knowledge that is sufficient to draw a reasonable working conclusion without having to know the processes in detail at the molecular level, as the term mechanism might imply."
Draft Dioxin Reassessment, Part III. Integrated Summary and Risk Characterization for 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and Related Compounds U.S.EPA, 2000b. page 41.	mode	To support the cancer risk assessment of 2,3,7,8-TCDD and related compounds.	One aid to the use of more information in risk assessment has been the definition of mode versus mechanism of action. Mechanism of action is defined as the detailed molecular description of a key event in the induction of cancer or other health endpoints.
Guidance for Identifying Pesticide Chemicals and Other Substances That Have a Common Mechanism of Toxicity. U.S.EPA, 1999. page 4.	mechanism	To identify chemicals that will be modeled by dose additivity based on common action.	Common mechanism means the same, or essentially the same, sequence of major biochemical events such that the underlying basis of the toxicity is the same, or essentially the same.
Mileson, B. E.; Chambers, J. E.; Chen, W. L.; et al. Common mechanism of toxicity: a case study of organophosphorus pesticides. Toxicol Sci. 1998 Jan; 41(1):8–20.	mechanism	To identify chemicals that will be modeled by dose additivity based on common action.	"Common mechanism is described as the major steps leading to an adverse health effect following interaction of a pesticide with biological targets. An understanding of all steps leading to an effect is not necessary, but identification of the crucial events following chemical interaction is required to describe a mechanism of toxicity." Common mechanisms means (a) cause the same critical effect, (b) act on same molecular and tissue target, (c) act by same biochemical mechanism and possibly share a common toxic intermediate.
Guidance for the preparation of an interaction profile. ATSDR, 2001b, pages 26–39. Guidance manual for the assessment of joint toxic action of chemical mixtures, ATSDR, 2001a, page 8.	mechanism	To choose a model of joint toxic action.	Should include information on events occurring at the molecular or receptor site level and at higher levels of biochemical, physiological, or pathogenic activities, such as toxicological response in the whole animal. Dose additivity means that chemicals behave as dilutions of one another, differing only in potency, and DRCs are parallel.

definitions, but Guidance for Conducting Health Risk Assessment of Chemical Mixtures (U.S.EPA, 2000b) uses the terms interchangeably (Table 1). EPA's guidance document for identifying chemicals with common mechanisms of toxicity (U.S.EPA, 1999) offers no clear definition of either term, but uses mechanism to refer to the same concepts defined as mode by other documents (Dellarco and Wiltse, 1998; U.S.EPA, 2001) (Table 1).

To summarize, "mode" and "mechanism" include different sets of mechanistic information: mechanism is detailed, stepwise information at various levels of biological organization; mode includes only the critical mechanistic steps that produce a characteristic biological effect. However, the terms are sometimes used interchangeably, and there would appear to be no consensus strategy or universal concept for categorizing modes of action, either pharmacologically or toxicologically. Regulatory guidance documents on mixtures are similarly inconsistent, further increasing the difficulty of understanding the level of mechanistic detail needed to predict the toxicity of chemicals in mixture. Therefore, when determining similarity between chemicals in a mixture, chemicals may not appear adequately similar to support use of an addition model when looking for a similar mechanism of action, but may appear adequately similar when looking for a similar mode of action. Despite terminology, however, it should be possible to decide the level of mechanistic information necessary to categorize chemicals for mixture risk assessment providing there are clear criteria for assessing the strength of the available data required for the intended purpose.

Proposed criteria for evaluating mode or mechanism of action

Three different sets of criteria are applied by government agencies in the US to evaluate mechanistic data for use in risk assessment. The most stringent of these is found in EPA's guidelines for carcinogen risk assessment, which we refer to as the "threshold dose response criteria" because they are required to differentiate threshold from non-threshold carcinogens. Here, EPA stops short of requiring a full characterization of 'mechanism' of action (a description of each molecular event in the pathway to toxicity), but does require establishing the mode of action, which they define as mechanistic information from several levels of biological organization (i.e., a detailed pharmacodynamic characterization), characterization of the metabolism and distribution of the chemical in the organism or ecosystem (i.e., a pharmacokinetic characterization), and fulfillment of the Hill standards to demonstrate a causal connection between the mechanistic steps (Dellarco and Wiltse, 1998; Schlosser and Bogdanffy, 1999; U.S.EPA, 2000b, 2001).

U.S.EPA (1999) requires different and less stringent criteria in their guidelines for identifying chemicals that share a common mechanism of toxicity for non-cancer endpoints. The term "mechanism of toxicity" is used to define these guidelines, but requires only that pesticides and other chemicals produce the same toxic effect by the same inducing major biochemical events. EPA contends that similar pharmacokinetics and metabolism strengthen the conclusion that chemicals share a common mechanism, but does not require this information to apply dose addition for risk assessment (1999). An International Life Sciences Institute (ILSI) expert panel convened to review EPA's proposed common mechanism guideline and concluded that chemicals should be considered to have the same mechanism of action if they cause the same critical effect, act on the same molecular target at the same target tissue, and act by the same biochemical mechanism of action, possibly sharing a common toxic intermediate (Mileson et al., 1998). We refer to these as the "common mechanism criteria."

In their recent guidance documents on chemical mixtures and interactions, ATSDR (2001a, 2001b) and U.S.EPA (2000a, 2000b) do not offer explicit criteria for deciding whether chemicals share a common mechanism. However, both suggest that target organ similarity is a sufficient basis for using dose addition to assess risks posed by chemical mixtures. We refer to this as the "target organ criterion." Here, U.S.EPA (2000a, 2000b) advises that establishing a similar mode of action is a stronger basis for applying dose addition than simply demonstrating similar target organs, but does not require a toxicologic comparison beyond target organ effects.

Sufficiency of the criteria to predict dose or response addition?

All three sets of criteria purport to define the "mode of action" rather than the "mechanism of action," despite the terminology that may appear in title of the guidance document. Regardless, the critical question for risk assessment is not terminology, but whether these sets of criteria form a sufficient basis for predicting mixture toxicity. Several examples illustrate why none of these sets of criteria lead to an adequate mechanistic characterization for predicting mixture toxicity.

It is well known that chemicals may affect the same target by a multitude of mechanisms, some of which may produce significant interactions rather than dose addition. Many examples exist, such as protection of acetaminophen hepatotoxicity by pretreatment with the hepatotoxicant clofibrate (Chen et al., 2002; Nicholls-Grzemski et al., 2000), a combination that is quantitatively antagonistic. Although U.S.EPA (2000a, 2000b) and ATSDR (2001a, 2001b) discuss the advantage of identifying similar mechanisms for chemicals that have the same effect in the same target organ, the lack of a requirement for this information would seem to argue against using target organ as the sole criterion for a dose addition default model in mixture risk assessment.

The common mechanism criteria proposed by U.S.EPA (1999) and ILSI (Mileson et al., 1998) are slightly more comprehensive, requiring data on target organs, target molecules, and underlying biochemical pathways. These criteria appear to be supported by the results of in vitro studies that demonstrate dose addition between acetylcholinesterase-inhibiting organophosphorus pesticides (Richardson et al., 2001), but may not be adequate to provide a prediction of dose addition in vivo or between different structural classes of acetylcholinesterase inhibitors because they do not require a characterization of pharmacokinetic behavior and metabolism. Even among a class of chemicals that share a common mode of action, the prediction of dose addition in vivo is complicated at low doses (NOAEL and below) due to the potential for differential metabolism (activation or detoxification) of various compounds and differential metabolism among different organisms (e.g., test versus target). Indeed, alteration of pharmacokinetic behavior and interference with metabolism appear to be the most common determinants of drug and chemical interactions (Krishnan and Brodeur, 1991).

Although the importance of pharmacokinetics is often discussed at the physiological level, kinetic differences can dictate the combined action of two chemicals at the level of the target enzyme itself (Jackson, 1993). A particularly relevant example is the reduction in toxicity for some organophosphorus compounds observed with carbamate pretreatment. Carbamate insecticides and many organophosphorus compounds act via reversible inhibition of acetylcholinesterase, but the kinetics of reversal is more rapid for the carbamate-acetylcholinesterase complex. Depending on the timing of exposure, this subtle kinetic difference can produce a competitive antagonism (Hayes and Laws, 1991). Differences in the pathways of biotransformation may also dictate whether two chemicals exhibit additive or interactive toxicity in mixture. For example, two organophosphorus pesticides that share the same detoxification pathway might be dose additive at concentrations both above and below their respective NOAELs because the chemicals would both contribute to saturating one detoxification capacity. However, for organophosphorus compounds that are detoxified by different esterases with different saturation capacities (for a review, see Hayes and Laws, 1991), the combined effects might be independent at concentrations below the respective NOAELs.

Because NOAEL-dependent dose addition probably occurs for many groups of chemicals, the common mechanism criteria may be broadly inappropriate for many types of mixtures. For example, hydrogen sulfide and cyanide both form methemoglobin adducts and both prevent the utilization of molecular oxygen in cellular metabolism by inhibiting electron transport in the mitochondrial cytochrome oxidase complex (Nicholls and Kim, 1982; Smith et al., 1977). These molecular events lead to the same pattern of clinical toxicity for both chemicals (Smith, 1991). Thus, these chemicals fulfill all the common mechanism criteria and, on that basis, would be predicted to be dose additive in mixture. Nonetheless, a detailed knowledge of metabolism leads to a different conclusion. Hydrogen sulfide is detoxified principally by sulfide oxidase, a high efficiency, low capacity enzyme that coverts hydrogen sulfide to thiosulfate. In contrast, cyanide is detoxified principally by rhodenase, an enzyme that utilizes thiosulfate to convert cyanide to the relatively nontoxic thiocyanate (Fig. 2). Increased thiosulfate concentrations drive this conversion and accelerate the detoxification of hydrogen cyanide, so effectively, in fact, that exogenously administered thiosulfate is a clinical antidote for hydrogen cyanide poisoning. Thus, detoxification of hydrogen sulfide to thiosulfate effectively raises the toxic threshold of hydrogen cyanide and their combination would be antagonistic at doses below the saturation capacity of rhodenase. Dose addition would not be predicted until the concentration of each chemical exceeds the metabolic capacity.

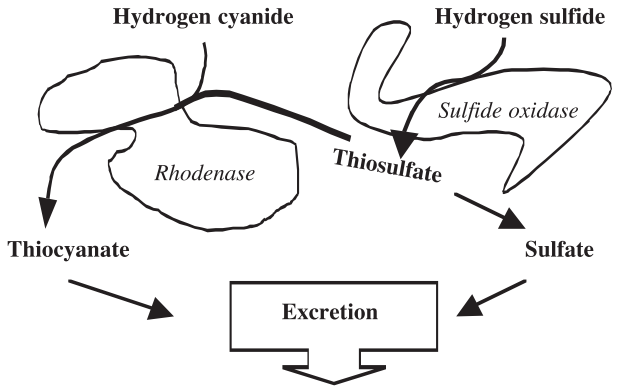

Fig. 2. Diagram of the detoxification pathways for both hydrogen sulfide and cyanide is shown, emphasizing the use of the thiosulfate intermediate produced by hydrogen sulfide metabolism in the detoxification of cyanide.

The short-chain aliphatic alcohols represent yet another example that calls into question the utility of the common mechanism criteria for predicting dose addition in mixtures. Methanol, ethanol, normal propanol, isopropanol, and the various isomers of butanol are well-studied, structurally related chemicals whose general neurotoxic effects are thought to be due to direct physical–chemical action that perturbs membrane fluidity (van Wezel et al., 1996, 1997), induces conformational changes in ion pores and membrane receptor proteins secondary to effects on membrane fluidity (Charney et al., 2001; Wimer et al., 1983), and activates inhibitory gamma amino butyric acid (GABA) receptors and stimulatory NMDA glutamate receptors in the central nervous system (Fleming et al., 2001). These alcohols are also metabolized by similar pathways and may compete for the same metabolic enzymes. Thus, these chemicals have mechanistic similarities even beyond those required by the common mechanism criteria. Nonetheless, dose additive toxicity is not observed in humans because the human optic nerve is particularly sensitive to formaldehyde, a toxic metabolite of methanol, but is less sensitive to acetaldehyde,

the corresponding metabolite of ethanol. Rather than being dose additive, ethanol antagonizes the retinal toxicity of methanol by competition for metabolic enzymes. In fact, ethanol is used clinically as a treatment to prevent blindness in methanol-poisoned humans.

The four examples presented above raise serious questions about predicting dose addition in mixtures from common modes of action as defined by either the target organ or the common mechanism criteria. Kinetic parameters, metabolic detoxification, and the sequence and relationship between mechanistic steps are absent from these sets of criteria, but may be critical for predicting the combined action of chemicals in mixtures. These mechanistic factors are encompassed by the threshold dose-response criteria proposed by U.S.EPA (2001) for identifying chemicals with carcinogenic dose thresholds. Because understanding the dose response curves of individual chemicals is critical for understanding the combined action of chemicals in mixtures (for a review, see Borgert et al., 2001), it would seem that EPA's threshold dose-response criteria are more appropriate for mixtures than either the target organ criteria (ATSDR, 2001a, 2001b; U.S.EPA, 2000a, 2000b) or the common mechanism criteria (U.S.EPA, 1999), even though they were not developed for the purpose of mixture risk assessment. Nonetheless, all three sets of criteria consider only the modes of action for single chemicals rather than the mechanisms by which chemicals might interact. A scheme for considering potential mechanisms of interaction has not been proposed to our knowledge, but we believe such a framework should be given serious consideration.

Is it practical to categorize chemicals by their mode of action?

The feasibility of obtaining and interpreting the requisite mechanistic data is critical to evaluating whether mechanistic approaches are likely to improve the scientific basis of mixture risk assessments (Borgert, in press). Although identifying the mode of action, as proposed in the various criteria, is less demanding than characterizing the complete mechanistic sequence, even this protracted set of data is likely to be difficult to obtain and interpret for most chemicals. Potential difficulties include the following:

- Target organ effects have not been identified for all chemicals;
- There is sparse mechanistic data for many chemicals, making characterization of the mode of action uncertain;
- Many chemicals produce different effects in different dose ranges, making the characterization of mode highly dependent upon dose;
- Many chemicals cause multiple effects by different mechanisms, even within the same dose range, obscuring causal links between key mechanistic steps and effects;
- Pharmacokinetics are poorly understood for many chemicals, especially below the observed effect range.

One could argue that the mechanistic data required to overcome these difficulties could be obtained by simply increasing the funding devoted to mechanistic research or requiring the data through regulations. Certainly, mechanistic research has become the cornerstone of the pharmaceutical and pesticide industries and these industries have generated a great deal of mechanistic information. However, it is important to understand that these industries require chemicals that produce a single desired effect at a dose well below that which produces undesired effects. This requirement can only be met by chemicals that exhibit potency, specificity, and selectivity of action across many levels of biological organization, including molecular, biochemical, tissue, and organ levels, and the population level for pesticides. Furthermore, it is desirable for drugs and pesticides to be detoxified and eliminated by a very few metabolic pathways to minimize inconsistent responses due to interindividual variability in metabolism.

Interpretable mechanistic information can be generated much more readily for chemicals that are specific, selective, and potent than for chemicals that are not. The reason is quite simple; it is scientifically much easier to elucidate a discreet mechanistic pathway for a potent chemical than to tease apart multiple mechanisms that occur within a similar dose range. Industrial chemicals and consumer products, however, are not developed for their ability to produce biological activity and in fact, the goal is to avoid it. Therefore, it is not surprising that these chemicals usually fail to exhibit significant specificity, selectivity, or potency by a relatively discreet mechanistic pathway. For this reason, merely increasing financial commitments or regulatory requirements is unlikely to increase the availability or interpretability of mechanistic data for industrial chemicals and consumer products.

The difficulty of obtaining interpretable mechanistic data is apparent in the fact that a detailed mechanistic understanding has remained elusive even for several widely used and extensively studied drugs, including general anesthetics (Baldessarini, 2001) and some antipsychotic agents (Beattie, 2001). For both anesthetic gases and antipsychotic drugs, the integrated effect of several different mechanisms appears to produce the clinical response rather than a single, discreet, readily identifiable mechanistic pathway. Because several mechanisms appear to operate at once in producing the clinical effects of these drugs, it is difficult to link specific effects with specific mechanistic pathways. Consequently, it is impossible to categorize such chemicals according to a discreet mode of action, even with data obtained at clinical exposure levels that generate overt effects. This does not bode well for efforts to identify and categorize modes of action for substances when exposures are at much lower environmental levels, near or below NOAELs.

Categorizing the mode of action can be difficult even for chemicals that are closely related structurally. For example, all of the short-chain aliphatic alcohols are central nervous system depressants; defat the skin with prolonged dermal contact; are respiratory and ocular irritants at high airborne concentrations; produce fatty liver with prolonged dosing; and are reproductive and developmental toxicants at high doses (Wimer et al., 1983). Subchronic and chronic toxic effects are thought to occur by modes of action different than those responsible for neurotoxic effects (discussed in the previous section). Disruption of lipid metabolism results in a reversible increase in circulating triglycerides, persistent hyperlipidemia, and accumulation of triglycerides in liver (alcoholic fatty liver) with subchronic administration, in addition to cirrhosis with chronic high-dose administration. Inhibitory effects on cell proliferation and migration (Miller, 1986; Miller and Potempa, 1990) and on neuronal differentiation (West et al., 1986) may be linked to interactions with cyclic AMP and cAMP-dependent protein kinases (Pennington, 1990; Shibley et al., 1997). Fetal alcohol syndrome has been proposed to result from an indirect effect on maternal zinc metabolism and nutritional imbalances that disrupt placental nutrient transfer (Dreosti, 1993). The variety of mechanistic pathways possible for these structurally similar toxicants and their exposure duration-dependence illustrates the difficulty of categorizing chemicals by their mode of action.

Another reason that it may not be possible to categorize chemicals by their mode(s) of action is the potential for exposure duration and dose-level to affect both the mechanism and the response. This is particularly true when complex physiological systems are responsive to the action of the chemical, as occurs during the complex and often unpredictable physiological changes following administration of nicotine. Nicotine produces an array of different physiological effects through pharmacodynamic actions on a variety of neuroeffector and chemosensitive sites in the body. These actions include both stimulation and desensitization of cholinergic receptors at autonomic ganglia and skeletal neuromuscular junctions. Nicotine increases heart rate by excitation of sympathetic ganglia or by paralysis (desensitization) of parasympathetic ganglia, and, nicotine slows heart rate by paralysis of sympathetic ganglia or by stimulation of parasympathetic ganglia. The pharmacological picture is further complicated by nicotine's chemostimulatory effects on the carotid and aortic bodies and medullary centers of the brain that affect heart rate, as well as by the compensatory cardiovascular reflexes resulting from blood pressure changes induced by nicotine. Finally, nicotine also stimulates the release of epinephrine from adrenal glands, an effect that stimulates heart rate and increases blood pressure.

Despite understanding the complex pharmacology of nicotine, it is extremely difficult to place its mode of action into one or even a few categories, much less to predict whether combinations of nicotine and other cholinergic agonists will have an overall stimulatory or inhibitory effect on the cardiovascular system. A number of complex questions would arise. Do other cholinergic agonists act as partial agonists or antagonists with nicotine, and if so, which effects would be partially antagonized? Do combined effects of cholinergic agonists and nicotine on heart rate and blood pressure result from pharmacodynamic interaction at cholinergic receptors or indirectly via affects on the release of adrenergic hormones? Does desensitization by nicotine blunt the effects of other cholinergic agonists administered concomitantly? Does the timing of administration affect the response to a combination of nicotine and other cholinergic agonists?

As is true for combinations of nicotine and other cholinergic agonists, it will often be difficult to predict whether the combined action of chemicals with similar modes of action would increase (i.e., dose addition, response addition, synergy), decrease (i.e., antagonism), or result in no change (response addition) for any particular toxic effect under consideration. Calabrese (1991), in his excellent reference text entitled "Multiple Chemical Interactions," summarizes the difficulty succinctly:

> Since most toxic substances have multiple toxic effects, the nature of any chemical interaction may vary depending upon the response that one measures. For example, since chlorinated insecticides and halogenated solvents produce liver injury independently, they may be reasonably expected to act in an additive or synergistic manner when combined. However, the insecticide is likely to be a central nervous system stimulant, when the solvent may be a central nervous system depressant. Thus, their joint action may result in an antagonistic response (Murphy, 1980, 1983).

What data support that a common mode of action predicts dose addition?

Regardless of the theoretical and practical difficulties that arise in attempting to predict mixture toxicity from the modes of action of constituent chemicals, the strengths and weaknesses of the supporting (or contradicting) data should ultimately be used to evaluate the approach. The extent and consistency of the data should be considered as well as whether any studies have directly tested the hypothesis that dose versus response addition can be reliably differentiated based on a defined set of criteria or defined measure of mechanistic similarity or dissimilarity.

Only a few publications address the most appropriate model for combined action of chemicals in mixtures, but most of those do not rigorously distinguish chemicals by mode or mechanism of action. Pozzani et al. (1959) reported that the toxicity of only 2 of 36 randomly selected pairs of vapors departed from dose addition by more than 1.96 standard deviations, almost exactly the number expected based on a 95% confidence interval. This study could be

interpreted as support either for the assumption of additivity or for relatively small antagonistic and synergistic effects among vapors, when they occur. It is unclear from the Pozzani report whether the statistical power of the assays could detect even large departures from the predicted additive effect. Consistent with the latter interpretation, Smyth et al. (1969) reported that among all possible combinations of 27 chemicals chosen at random, no pair departed from predicted additive effects by more than five-fold. The vast majority of pairs deviated from additivity by less than two-fold. Ikeda (1988) surveyed studies published in 20 leading environmental and occupational toxicology journals and found that among 55 reported chemical interactions, 38 were classified as less than additive. Those interactions classified as greater than additive generally occurred at doses higher than would be received from actual environmental or occupational exposures. Ikeda concluded that additivity is reasonably protective for exposures to mixtures of chemicals with similar or dissimilar action. While supporting the general concept that dose addition is a conservative assumption for pairs of chemicals, none of these studies (Ikeda, 1988; Pozzani et al., 1959; Smyth et al., 1969) directly tested whether the combined action of two chemicals can be correctly predicted based upon mechanistic data, nor whether dose addition is more applicable than response addition at low concentrations.

More recently, Feron et al. (1995) studied a variety of different chemicals with "similar" and "dissimilar" modes of action and concluded that the effects of mixtures were generally less than additive, with nasal irritation caused by mixtures of aldehydes conforming to competitive agonism rather than additivity. Additivity did, however, appear to be applicable for increased organ weights produced by nephrotoxicants that shared the same mode of action. Although the assessment was somewhat unclear, other nephrotoxic effects including histopathology, enzyme and protein markers, concentrating ability, and excretion of glucose did not appear to be dose additive. Jonker et al. (1996) examined the additivity assumption for mixtures of nephrotoxicants, again concluding that chemicals with dissimilar modes of action were less than dose additive but that chemicals with the same mode of action were dose additive. Neither of these studies, however, explained the criteria used to conclude that two modes of action were "similar." The authors subsequently recommended caution when inferring additivity at low doses from high-dose animal studies because less than additive effects may predominate at levels significantly below the NOAEL (Groten, 2000).

Consistent with Groten's (2000) caution, results in FETAX show that combinations of osteolathyrogens with similar mechanisms of action are generally dose additive or less than dose additive when both chemicals are present at concentrations that produce equal numbers of malformations (Mentzer et al., 1999; Poch and Dawson, 1996). However, as the concentration approaches a no-effect level, even for one chemical, the combined response more closely conforms to independence. Combination responses greater than dose additive have not been observed, and only a few responses significantly less than response additive have been observed. These results imply that dose addition may be a conservative assumption for the combined osteolathyrogenic effects of chemicals when they are present at concentrations above their NOAELs, but that independence becomes more predictive when the concentrations of the component chemicals are below their individual NOAELs.

One potential reason for low dose mixtures being less than additive is that the mode of action could be different below the NOAEL. Hermans et al. have performed the most thorough investigations to date of the additivity assumption for low dose mixtures of aquatic toxicants. They found that mixtures of organic chemicals at low concentrations generally exhibit concentration addition (Loewe additivity) for narcosis on either an acute or chronic basis, regardless of the modes by which the chemicals produce toxicity at higher concentrations (Hermens et al., 1984, 1985). McCarty and Mackay (1993) interpret these data as indicating that when chemicals are present in a mixture at concentrations below 0.3–0.02 times their threshold for a specific toxicity, their combined action does not occur as a consequence of the specific mechanism for that toxicity. Instead, at concentrations below their threshold for specific toxicity, organic chemicals merely contribute to an overall nonspecific narcotic effect by simple concentration (dose) addition. The narcotic effect of such mixtures in aquatic systems is best predicted from whole body doses received (critical body residues) (McCarty and Mackay, 1993). When multiple mechanisms are possible for a set of chemicals, comparing their potencies for each mode of action, as shown by Freidig et al. (1999), may be necessary if the goal is to choose the best model of combined action.

Because so few studies have directly tested the hypothesis that dose addition can be reliably differentiated from response addition based on a defined measure of mechanistic similarity or dissimilarity, it is difficult to draw firm conclusions. Nonetheless, some generalities can be stated based upon what is known of mechanisms of drug actions and interactions. First, chemicals rarely operate by a single mode of action, so ascribing a toxic effect to a single mode of toxicity may not usually be possible. Chemicals in mixture can produce different types of combined action depending on their concentrations and relative concentrations; thus, a global characterization of combined action for even a single pair of chemicals—one that holds for all ratios and concentrations of the chemicals—requires experimental confirmation.

Although receptor-based mechanisms are often cited as conforming to dose addition, the pharmacological experience has been that mixed receptor agonist or antagonist properties often produce combined effects that are less than dose additive. For example, tamoxifen is a drug that binds and activates the estrogen receptor in several estrogen-responsive tissues and would be classified as having the same mode of action as estradiol based on the level of mechanistic data

available for most chemicals. However, tamoxifen is not only less than additive in combination with estradiol, but also antagonizes estradiol in breast tissue, an effect that makes it efficacious in the treatment of estrogen-receptor-positive breast cancer (Wakeling, 1995). Non-steroidal anti-inflammatory agents are another class of compounds that have similar modes of action, yet these drugs are not prescribed together because their combination decreases efficacy. In fact, therapeutic regimens rarely involve co-treatment with agents that have the same mechanism of action, even though theoretically, such protocols have the potential of increasing therapeutic efficacy without increasing toxicity.

Finally, it should be recognized that while an empirical demonstration of dose addition has classically been used to infer common modes of action, the reciprocal statement cannot be made. This distinction is important. While it is possible to test empirically for dose addition versus response addition, concluding that two chemicals share a mode or mechanism of action requires a series of complex professional judgments. The conceptual bases of these judgments have not been agreed upon within the scientific community, as described earlier in the discussion presented here. Using interaction experiments to identify chemicals that act via the same or different modes of action is classical hypothesis testing that might yield data directly pertinent to the risk assessment of chemical mixtures.[2] In contrast, sets of criteria used to make determinations about mechanistic similarity should be tested empirically before they are accepted.

From a scientific standpoint, the demonstration of dose addition would be an important test of whether a set of mechanistic features is actually useful in predicting the combined action of two or more chemicals. Combination studies could help define the mechanistic criteria necessary to predict combined action for various types of chemicals and various types of effects. Clearly, questions such as these will remain unanswered unless research is focused on determining how much mechanistic information is required to predict combined action and whether the same amounts and types of mechanistic information are useful for all chemicals and all forms of toxicity. The scientific basis of mixture risk assessments could be improved markedly by increased emphasis on answering these and related questions.

Conclusions

Categorizing chemicals by ‘‘mode’’ or ‘‘mechanism’’ of action to predict dose response characteristics for mixture risk assessments and product safety assessments is premature until the scientific community reaches consensus regarding which concept is to be used. Specifically, the amount and types of mechanistic information necessary to predict combined action must be clarified before the recommended approach is useful. Criteria for evaluating the mechanistic data set for chemicals should be more rigorously defined and should be consistent between guidance documents that address threshold and combined action. Predictions based on mechanistic similarity may simply be impractical for most chemicals due to uncertainties in the mechanisms or modes of action by which they operate. Obtaining the required mechanistic information may be technically impossible for chemicals that produce effects by multiple mechanisms.

The database to support mechanistic similarity as a determinant of dose addition is sparse and equivocal. Thus, it is premature to assume dose addition for chemicals that appear to be mechanistically similar and to response addition models only for chemicals that appear to be mechanistically dissimilar. Because these simple models were developed for binary mixtures, their applicability to more complex mixtures is quite uncertain. Dose addition should be correlated with specific mechanistic features for particular toxic effects before the approach is generalized. Default assumptions should be reconsidered, particularly for extrapolating the assumption of dose addition to concentrations of chemicals below their no effect levels. Currently, there are insufficient data to support or refute this assumption generally. The possibility for NOAEL dependence of dose addition should be considered in the assessment process, and a default assumption of response addition considered for levels below some point of departure (e.g., doses less than 20% the ED_{01}).

Considerable basic research will be required to understand how mode of action for individual chemicals is related to the toxicity of chemical mixtures. As described earlier for hydrogen sulfide and cyanide, the potential for NOAEL-dependent shifts in combined action (e.g., from interaction to non-interaction) underscores the need for basic research aimed at defining the dose-dependent transitions in mechanism of action that occur near or just below the NOAEL. Detailed dose-response characterizations at various levels of biological organization may be required to help identify whether molecular, cellular, or physiological endpoints are most predictive of such transitions. Given that most toxicologic interactions have a pharmacokinetic basis (Krishnan and Brodeur, 1991), it may be most productive to study how pharmacokinetic parameters change with dose and with the presence of other chemicals to identify the mechanistic steps that might be most predictive of mixture toxicity.

It is especially important in mixture toxicology to distinguish between policy decisions and science-based decisions. The assumptions used in mixture risk assessment may have profound inherent uncertainties, especially for mixtures of chemicals at concentrations below their currently acceptable levels as discussed in this paper. Extensive research is needed before scientifically defensible general theories and general conclusions can be reached regarding the use of mechanistic information for predicting combined action, especially at low

[2] For an excellent example of this approach, the reader is referred to a series of publications on osteolathyrogens in *Xenopus* (Dawson, 1991; Dawson and Poch, 1997; Dawson and Wilke, 1991; Dawson et al., 2000; Mentzer et al., 1999; Poch, 1993; Poch and Dawson, 1996).

concentrations. Until a scientifically defensible, generally applicable theory for mixtures is formulated and a sufficiently broad base of data directed toward examining this theory is generated, regulatory approaches that utilize mode of action to predict mixture toxicity will remain tenuous.

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