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Support of Science-Based Decisions Concerning the Evaluation of the Toxicology of Mixtures: A New Beginning

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INTRODUCTION

Evaluation of potential human health hazards from exposure to chemical mixtures in the environment presents one of the most difficult challenges for risk assessment as well as for toxicological research. Yet legislative mandates (the so-called Superfund Act of 1980, the Food Quality Protection Act, and the Safe Drinking Water Act, Amendments passed in 1996) that apply to the U.S. EPA require consideration of joint chemical exposures and of chemical mixture toxicity in regulatory decision-making.

Current methods for conducting chemical mixture health risk assessments were developed to use available experimental data as well as the health effects data in the toxicological and epidemiological literature. These methods generally rely on default assumptions whose validity is unknown (ATSDR, 2000a,b,c; U.S. EPA, 2000). Moreover, the basic toxicology database is inadequate for assessing risk for the vast majority of chemical mixtures. Thus, a substantially enhanced toxicology research program is required in order to provide a strong, science-based approach to the assessment of the potential toxicity of chemical mixtures.

Chemical mixture risk assessment methods fall into the two general categories of whole mixture approaches (in which complex mixtures are evaluated as though they are single entities) and component-based approaches (in which the interaction of certain individual components in a mixture is considered to estimate toxicity of the mixture) (NRC, 1988; Cassee *et al.*, 1998; Calabrese, 1991; Yang, 1994). Whole mixture approaches involve either direct evaluation of the mixture of concern or an assessment of the mixture of concern using data available on a "sufficiently similar" mixture (i.e., one that has similar components and proportions of those components to the mixture of concern). The most widely used component-based methods are dose addition (assumes same mechanism of action across components) and response addition (assumes independence of toxic action across the components). Dose addition operates by summing the exposure levels of similar components in a mixture and estimating mixture risk directly from the summed dose. Response addition operates by estimating risk for each individual component and summing these to estimate the mixture risk. Methods have also been proposed for incorporating evidence of toxicologic interactions (Hertzberg *et al.*, 1999; Mumtaz and Durkin, 1992). It is recognized that the understanding of the mixture must be improved to reliably assess and predict specific human and environmental risks from chemical mixtures.

There is often a discussion of the differences between interpreting the effects of exposure to mixtures for wildlife and humans. Issues relative to the effects of interactions and their effects on extrapolations and testing methods are similar for wildlife and humans. Thus, there are few issues relative to the approaches to studying mixtures that are unique to wildlife species. Therefore, no effort has been made to separate these issues in this document. Although each of the points made in this report can be applied to both humans and wildlife, there are differences in exposure pathways. Because wildlife species are often exposed to complex environmental mixtures and there are a number of similarities in biochemical pathways between wildlife and humans, they can serve as environmental sentinels (Kendall *et al.*, 1998). However, additional research is

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needed because there can be significant differences, especially in exposure pathways, xenobiotic metabolism, and physiology.

Under the leadership of the Society of Toxicology, in conjunction with the Society for Environmental Toxicology and Chemistry, a Steering Committee[1] was formed to convene an Expert Working Group (the authors of this Commentary) to discuss the challenges of mixture toxicity and suggest research strategies to meet policy needs.[2] This Commentary represents a consensus paper suggesting that, while there is a need to build upon the current science, toxicology must advance into largely uncharted territory that places a strong emphasis specifically upon three key ideas:

1. Toxicology experiments on whole mixtures or mixture components should include doses at or below the no-observed-effect levels [NOAELs/NOELs] for individual mixture components. The mixture components that are tested and their relative proportions in the mixture also should reflect those seen in environmental samples. In addition, the impact of the unidentified materials in the mixtures should be considered.
2. Amplify results generation and conserve resources through collaborative efforts. Future experiments using a multidisciplinary team approach should be encouraged. Collaborative research using a program-project approach should also be encouraged in which multiple investigators plan the research, share tissues from a pool of commonly treated animals, and share biomathematical models and data analysis tools.
3. Employ novel approaches and new technologies. Cutting edge research approaches (e.g., computer modeling of interactions, new *in vitro* assays, efficient experimental designs) and methodologies (e.g., on genomics, proteomics, bioinformatics) should be applied to the mixtures issue. The information necessary to develop biologically based models should be generated.

[1] Steering Committee members (listed alphabetically): J. Bucher, National Institutes of Environmental Health Sciences (NIEHS); J. Bus, American Chemistry Council (ACC); W. Farland, U.S. Environmental Protection Agency, (U.S. EPA); J.Foran, Society for Environmental Toxicology and Chemistry (SETAC); J. Goodman (Chairperson), Michigan State University; S. Lamb (Society of Toxicology); A. Mason, Chlorine Chemistry Council (CCC); R. Parrish, SETAC; and C. Thompson, NIEHS.

[2] The SOT Steering Committee commissioned this Expert Working Group as the first effort in a series of activities intended to advance the scientific understanding of environmental mixtures as the foundation supporting future risk assessments. This Expert Working Group was charged with evaluating the state of the science on environmental mixtures, providing a conceptual framework for future mixtures research, and suggesting potential areas for empirical and mechanistic experimentation. Future SOT-led efforts will build upon this base and provide guidance regarding the scope of a research agenda, suggest relationships for conducting collaborative research, and provide scientific insights into the evolving policies addressing environmental mixtures.

Because the issues among the above three areas are interwoven, we selected the following aspects for expanded discussion in the hope of stimulating interests and thoughts in chemical mixture research. The SOT Expert Working Group concluded that the scientific challenges of chemical mixture toxicology and risk assessment are substantial and warrant considerable attention. In particular, they recognized a need to move mixture research beyond current scientific methods and practices in order to strengthen the mechanistic understanding of the potential toxicity of mixtures to improve the quality of risk assessments and to aid in the development of improved science policy.

FOCUS ON REAL WORLD EXPOSURES

The limitations of the available scientific databases for chemical mixtures challenge decision-makers. The majority of mixture studies in the available literature are experiments testing high doses of a few constituents, using experimentally expedient compositions. Most real world human and environmental exposures, however, are to low doses and to a complex range of chemicals. And perhaps of equal importance, most mixture toxicology studies have not considered the potential implications of simultaneous exposures to the broad range of natural chemicals intrinsic to human and animal diets. Thus, the toxicity of low-dose component mixtures has not been effectively characterized. Because of critical data gaps, uncertainties occur when extrapolating from high concentrations in the laboratory to lower environmental concentrations (reviewed in Berenbaum 1989; Borgert, 2001; Groten *et al.*, 2000) or from component information to complex mixture exposures. Such extrapolations are complicated by the influence that both dose and relative component concentrations can have on potential interactions and toxicity.

To improve on default approaches to risk estimation for mixtures, it is necessary to develop data that support risk calculations in a specific quantitative way. One of the key hypotheses to address is whether mixture toxicity at low concentrations is best represented by the most toxic component of the mixture or, conversely, by a model of combined toxic action. A few mixture studies have attempted to relate the toxicity of the mixture to the expected toxicity based on individual mixture components. Most notable for purposes of low-dose risk assessment are studies suggesting that when toxicologically dissimilar chemicals are present in a mixture near their minimum effect concentrations, the toxicity of the mixture reflects the toxicity of the most potent component of the mixture for any particular toxic endpoint (Jonker *et al.*, 1996, 1993, 1990). Whether this holds as a general rule is an important question for mixture research because the answer has profound implications for risk assessment.

Dose addition is often used to estimate cumulative mixture risk at environmental exposure levels based on the assumption that a constant relative potency between mixture components in a substantially higher dose range signifies a common mode of action. Although this is a reasonable default approach in the absence of more informative low-dose data, experimental confirmation of dose additivity in a high-dose range does not necessarily imply similar behavior at substantially lower doses. Real-world environmental exposures to mixtures involve exposure levels of individual components that are lower than their individual experimental thresholds for toxic effects. Methods that are commonly used to characterize dose addition in the observable range of overt effects (e.g., isobolograms) do not necessarily extrapolate to dose levels of mixture components that are well below their individual NOAELs for overt effects. However, there is a rich statistical literature on the assessment of dose addition, as well as the characterization of departures from dose additivity (response addition, synergism, antagonism, etc.), including guidelines for efficient but powerful experimental designs. Alternative statistical designs analysis methods are beginning to be used, such as (fractionated) factorial designs, ray designs, dose-effect surface analysis, and statistical testing for departures from additivity. These statistical models, useful in detecting and characterizing interactions among components in a mixture, are based on the fundamental concept that an interaction implies a change of slope of the dose-response curve of one compound in the presence of another. Researchers need to reevaluate the concepts of dose addition and response addition, as they may be unnecessarily restrictive and compartmentalized. Development of more generalized approaches for describing additivity and departure from additivity of mixtures of chemicals with particular emphasis on low-dose regions would be useful. These are needed to ensure that data collected on mixtures at subthreshold individual doses will enable validation of quantitative risk predictions produced by biomathematical models that link precursor effects to overt toxic effects.

Empirical approaches have also been used to examine the impact of low-level exposures to mixtures. One such approach is to concentrate a complex mixture to produce observable toxicity in laboratory assays. This has the advantage of testing both the known components and the unidentified fraction of the mixture in the assay. At the same time, these studies can be problematic because the chemical composition of the complex mixture is changed; thus, the toxicological properties of the mixture may be altered. However, depending upon the chemicals and concentrations of concern, whole mixture studies can be performed in ways that reduce concerns over extrapolating to higher or lower concentrations (Chapin *et al.*, 1989; Heindel *et al.*, 1994; Groten *et al.*, 1997). Thus, more empirical studies such as these are needed in order to test the validity of, and to enhance the predictive power of, mechanism-based and computational models.

These dose-response considerations not only apply to empirical mixture studies, but also to mechanistic ones as well. Mechanistic studies focused on low-dose exposures can provide a biological basis for phenomena such as dose addition or response addition, lending critical support to the primary default assumptions used in mixture risk assessments. Any toxicant is likely to induce some biochemical effects in the target organism, associated with either the specific target molecule or with pharmacokinetic factors such as detoxication mechanisms. It is these precursor biochemical alterations, which may or may not be "adverse" effects, which will be able to extend the dose-response curve to low concentrations of toxicants. Causal associations must be identified for the biochemical changes and associated overt toxicity. While the biochemical parameter measured may be a precursor several steps prior to the production of the specific toxic effect, the ideal biochemical marker would be one that is in the direct mechanistic sequence from absorption of a chemical to production of its specific effect, that is, not influenced by processes or inputs other than the action of the chemical. It is also necessary to understand that dose influences mechanism. Since biological responses occurring at high doses will not necessarily happen at low doses, research designed to explore the existence of thresholds for potential toxicity should be encouraged.

Finally, toxicologists should consider potential differences in exposure factors between environmental mixtures and laboratory exposures in designing their experiments. Exposures to mixtures typically occur by several exposure pathways (e.g., oral, inhalation, dermal) for chemicals present in air, soil, water, food, and commercial products. Because the potential exists for changes in chemical composition during fate and transport of a chemical mixture in the environment, there are significant uncertainties related to identifying the chemical composition of a mixture and evaluating its toxicity.

To be useful in regulatory decision-making, the laboratory toxicity data should be representative of the potential toxicity caused by the environmental exposure. Experimental paradigms characterizing only the interactions of chemicals at high doses relative to actual environmental exposures will not provide the necessary data to support scientifically informed health policy decisions.

USE COLLABORATIVE EFFORTS TO EXPAND RESULTS GENERATION AND CONSERVE RESOURCES

Future experiments should be conducted using a multidisciplinary team approach, including the expertise of

toxicologists, epidemiologists, statisticians, risk assessors, modelers, exposure experts, and other scientists. Because resources are scarce, studies should target the chemical mixtures, exposure routes, dose levels, and potential interactions of greatest value to a risk assessment issue. The goals of such studies can be established to answer toxicological questions relevant to a regulatory decision. Appropriately designed interactions studies that use a factorial design are generally large and expensive; thus, efficient experimental designs and statistical models can be employed to provide information on toxicity and interaction effects without implementing a full factorial design. Researchers should be cognizant of using study designs and sample sizes sufficient to achieve reasonable power to detect "biologically meaningful" interactions if they exist. Otherwise, claiming the null hypothesis of zero interaction when an interaction is not detected may be misleading.

The involvement of scientists from other disciplines whose research is relevant to toxicology should be encouraged in collaborative research. Such research can be performed using a program-project approach in which multiple investigators plan the research and share tissues from a pool of commonly treated animals. Such efforts should lead to more efficient research through improved experimental design while decreasing the number of animals used.

Vertical communication among scientists operating at different levels of the risk assessment process is essential. It is incumbent on those developing statistical methods and biomathematical models for risk estimation to make known to the experimentalists the specific data needs for improving the risk calculations. Similarly, it is incumbent on experimentalists developing data at low doses on precursor biological effects to inform those developing quantitative methods for risk assessment how their refined data can be used to improve quantitative risk estimation for overt adverse effects. The best way to improve on default approaches to risk estimation for mixtures is to develop data that inform risk calculations in a specific quantitative way. Such data will enable refinement of the overly simplistic use of dose addition or response addition to estimate mixture risk at environmental exposure levels.

EMPLOY NOVEL APPROACHES AND NEW TECHNOLOGIES

With the remarkable advances in cell and molecular biology in the last few decades, particularly the explosive progress of genomics, proteomics, and bioinformatics in recent years, the area of toxicology of chemical mixtures, as in many other biomedical fields, may undergo revolutionary changes. Conventional animal toxicology testing methods are inadequate for the evaluation of chemical mixtures because of the complexity and high demand on resources (Yang, 2000). Even more importantly, conventional animal toxicology testing methods are usually single time point determinations at terminal sacrifice; these methods are not designed to obtain quantitative information of the time-course fate of the chemical in the body (i.e., pharmacokinetics) and/or time-course receptor interactions or toxic responses (i.e., pharmacodynamics). Thus, even if toxicologic interactions are detected, the mechanistic bases for such interactions typically remain unknown. Given these limitations, novel approaches should be investigated, new technologies applied, and approaches should be devised to integrate newly emerging data into toxicological evaluations. New methods can include: (1) computational technology; (2) mathematical/statistical modeling; (3) mechanistically based, short-term toxicology studies; (4) the latest advances in cell and molecular biology methodologies; (5) new *in vitro* studies for screening mixtures toxicity; (6) approaches developed to understand and analyze data on genomics and proteomics (i.e., bioinformatics); and (7) technologies available in other disciplines beyond the normal toxicological boundaries such as engineering and computer science.

Given that exhaustively testing all mixtures of concern in the laboratory is impractical, predictive tools are needed to focus on specific exposures. Computational models describing the mechanisms by which mixture components interact have the potential to play this predictive role, though such models are not yet generally available. Given the diversity of mixtures in the environment, predictive capability will be needed for mixtures other than those that were studied in the laboratory and that were used to support development of the computational models. This means that purely statistical models will be of limited value, as statistical models are less useful than models of mechanisms when extrapolating outside the domains over which they were developed. Biologically based models, on the other hand, can be useful for extrapolation since these models focus on the mechanisms of action that underlie toxic effects. A key point to be made here is that models are most useful when they are developed side-by-side with laboratory studies, so that each can inform the other. When the understanding of the biological issues is sufficiently mature, and the formal (computer) model correspondingly mature, then the model can have value for prediction for situations that have not been examined in the lab. Any expectation that (mechanism-based) computer modeling will some day provide the answer to the problem of mixture toxicology must assume this maturity of the biologically based computer model. Progress is most likely, therefore, when the focus is on cooperative interactions between toxicologists interested in experimental studies of mechanisms, statisticians with expertise in experimental design and

analysis, developers of computer models (who can also be the experimentalists), and risk assessors (who may also be the experimentalists and/or modelers). This paradigm for the efficient integration of laboratory research, computer modeling, and risk assessment is applicable not only to the problem of mixtures but also more generally in toxicology (Conolly *et al.*, 1999).

Another inherent advantage of mechanism-based models is that they can naturally incorporate information on variations in model structure and in parameter values to account for age, sex, and genetic status. This incorporation is possible to the extent that there is a clear understanding of how age, sex, and genetic status affect the mechanism (or mode) of action. For example, age-dependent changes in body and tissue size, in blood flow rates, and rates of xenobiotic metabolism can readily be incorporated into PBPK models (e.g., O'Flaherty, 1994). Interindividual differences in genetic status could be represented by, for example, the presence or absence in the model of specific xenobiotic metabolizing enzymes. Once again, it should be emphasized that the limitation with respect to incorporating such information into mechanism-based models is not the development of the models per se, but in the relevance and quality of the information available for use in the models. Cooperative interactions among individuals with the requisite expertise will thus be required.

Finally, to understand the toxicology of chemical mixtures, basic mechanistic data need to be obtained. All of the new techniques and technology, including proteomics and genomics, along with computer modeling and application of PBPK modeling to the mixtures problem require basic mechanistic research as a foundation on which to base these models. The specific methods and techniques used to develop basic mechanistic data are certainly important. However, the questions should be hypothesis driven and mechanism of action should be incorporated into the methodology and techniques involved in assessing mixture toxicity.

CONCLUSIONS

In discussing the future of toxicological research to enhance chemical mixture risk assessment and policymaking, the SOT Working Group reached several basic conclusions that are meant to guide research efforts. Clearly, the toxicological database available for estimating chemical mixture health risks must grow in both size and sophistication in order to make a difference in the way risks are currently estimated and regulated. As "smarter" data and analyses emerge detailing mechanistic data, genetic information, computer modeling predictions, mechanistic modeling results, interaction effects, and the behavior of mixtures at low doses, methods for evaluating health risks can be improved, and regulations can be based on better science. Because of the complexity of the mixture problem, continued creation and discovery of new laboratory procedures, experimental designs, and approaches is essential for supporting scientific advances in mixtures toxicology.

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