Methods for Assessing the Effects of Mixtures of Chemicals
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1 Introduction, General Conclusions and Recommendations*

1.1 OBJECTIVES AND SCOPE OF THE STUDY

When chemicals are released to the environment, either indoor or outdoor, they immediately become mixtures with other chemicals or with their own degradation products. Sometimes the chemicals are mixtures before they are released, either in formulations such as an askarel (PCBs (polychlorinated biphenyls) and chlorobenzenes) or by containing manufactured impurities such as dioxin. Industrial and municipal effluents, gaseous or liquid, are usually complex mixtures upon release. Chemicals enter the environment from different sources, at the same or different times or locations, to form mixtures of unlimited numbers which vary in composition and concentration.

This great variety may be classified into three types of mixtures. The first type is created at a given time and place, and has a defined and constant composition. Such mixtures may result from chemical processes or from incineration and are not necessarily composed of compounds having similar chemical structures or properties. For many well-controlled processes the mixtures may have a relatively stable composition over time. Cosmetics and foods containing intentional food additives are examples of mixtures of this type. However, many minor changes in composition may occur which can be important toxicologically. Although large problems may exist, both from the analytical as well as from the toxicological viewpoint, the assessment of this type of mixture can be handled. When the composition of the mixture used in the product varies in time, analysis, especially for toxicological purposes, will be very difficult.

The second type of mixture is composed of compounds with similar physical and chemical properties, such as PCBs or dioxins. Because of differences in distribution and transformation pathways of the individual compounds, or because of variation of release, the composition of the mixture will change with time. Any estimate of exposure, therefore, has to be specified with regard to space

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and time and should consider not only the chemicals originally present in the mixture but also any products of chemical interactions. Changing the composition of mixtures will result in different exposures of biota with possibly different effects or incidence of effects.

The third type of mixture to which biota can be exposed are coincidental mixtures. These mixtures are composed of compounds that occur by coincidence at the time and place of environmental sampling. No similarity needs to exist between the individual components of the mixture. This coincidental presence may occur frequently or it may result from a single, accidental release.

All types of mixtures create problems for analytical and toxicological evaluation and for monitoring. For example, some of the most acute current problems for human health and environmental welfare are caused by mixtures so intractable that the mechanism of action has not yet been fully explained. Three common examples are photochemical smog, acid rain, and tobacco smoke.

In other less troublesome mixtures many of the components are not biologically active or are present in low concentrations, and these need not be evaluated. In some instances one or two components of a mixture predominate in toxicity so that the problem of estimating the exposure to, and effects of, the mixture is manageable. For such mixtures the initial qualitative or semi-quantitative analyses are especially important to determine the scope of the toxicological assessment that will be required.

Although the difficulties in assessing the effects of mixtures have been recognized, techniques have not been generally available to surmount them. From time to time various approaches to the problem have been proposed. For example, one approach is to determine the composition of the mixture and estimate roughly the total toxicity of the mixture by adding the toxicities of the individual components (multiplying exposure by a coefficient of toxicity).

Some grouping of individual chemicals may be possible to save resources and effort in monitoring and assessment. This leads to a 'tier' organization of testing or to a 'toxicological fractionation'; both these methods have been used in designing approaches to the assessment of mixtures. Recent advances in analytical and computational technology have made progress easier in some of the difficult areas of the testing and evaluation procedures.

There have been few attempts to define systematically the practicality and limitations of evaluating the biological effects of complex mixtures of chemicals; the present study makes such an attempt.

The approach has been to commission in advance a series of contributed papers, and then to assemble the authors to prepare a Joint Report. The Joint Report thus brings together the collective thinking of the participants of the Workshop and their conclusions and recommendations for future work to improve the methods for evaluating the effects of chemical mixtures. This section introduces the Joint Report and sets forth the general conclusions and recommendations of the Workshop.

Like the two previous projects (Vouk and Sheehan, 1983; Vouk *et al.*, 1985), this study has two components, one dealing with the effects of mixtures of chemicals on human health, the other with the effects of exposure to several chemicals on non-human biota. Section 7 concentrates mainly on the effects of mixtures on non-human biota although aspects of this subject are discussed throughout the sections. Two major methodological problems are considered: (1) how to interpret the results of tests using complex mixtures as they occur, for example, in food, water, or air; and (2) how to combine the results of tests made with single chemicals. Other subjects discussed in the Joint Report are: environmental and biological monitoring of mixtures; environmental transformation and metabolism of mixtures; mechanisms of combined action; epidemiology and human case studies; and effects of mixtures on animal and plant populations, communities, and ecosystems.

The second part of this volume contains individually authored contributed papers.

1.2 METHODS FOR ESTIMATING, PREDICTING, AND INTERPRETING HUMAN HEALTH EFFECTS FROM SIMULTANEOUS OR SEQUENTIAL EXPOSURE TO SEVERAL CHEMICALS

The term 'exposure' is used by different people in different ways. To avoid confusion the following definition is recommended.

> The exposure to a given pollutant is a measure of the contact between the pollutant and the outer or inner (e. g. alveolar surface or gut) surface of the human body. It is usually expressed in terms of concentrations of the pollutant in the medium (e. g. ambient air and food) interfacing with the body surfaces. Once absorbed through body surfaces, the pollutant gives rise to doses in various organs or tissues. Doses are measured in terms of concentrations in the tissues. Records of exposure and dose should include an indication of the time and frequency at which an individual is subjected to them. (UNEP/WHO, 1977)

As with single chemicals, human subjects can be exposed to mixtures of chemicals by several different routes: air, water, soil, food, pharmaceuticals, and other consumer products. The importance of different exposure routes is primarily determined by patterns of human behaviour. There can be large differences in exposures of individuals within a population, as well as differences between populations in different geographical locations. Some important variables that determine human exposure are:

(1) type of workplace;
(2) dietary habits;
(3) life-style (smoking, drinking, use of prescription and non-prescription drugs);

(4) location of home and workplace; and
(5) mode of travel to and from work.

All these variables may be modified by several additional characteristics such as culture, education, type of community and migration.

In addition to human behaviour, the properties of the mixture also affect the relative importance of different routes of exposure. For example, exposure to some compounds by some routes is likely, while for other routes it is not. Exposure to mixtures of PCBs through food can be high, while exposure by inhalation may be negligible. For one mixture several routes of exposure may be important and, further, for one component of a mixture (e. g. lead in combustion fuel) exposure may be through several routes (inhalation, food, and water).

The methods used for evaluating human health effects of exposure to combinations of chemicals include: (1) analyses of human cells, tissues and/or body fluids for the presence of specific chemicals; (2) clinical and laboratory procedures for detecting biological effects of chemicals in exposed individuals; (3) epidemiological methods for detecting the effects of chemicals in populations; and (4) studies conducted on animals as models.

1.2.1 Human Cells, Tissues, and Body Fluids

Available methods for detecting single chemicals or mixtures in human body fluids (e.g. blood, saliva, breast milk and semen), tissues, and excreta (urine, faeces, exhaled air) include physicochemical tests for compounds that are frequently encountered in the workplace and in the environment. In general, these methods, such as chromatography and spectrometry, are based on extraction, purification, and quantification of chemicals by procedures requiring comparatively sophisticated equipment. Hence their applicability to large numbers of samples is limited. In addition, although such methods can detect individual compounds, their results provide no reliable measure of the overall exposure to other compounds which may be present in a mixture, unless an effort is made to determine all or most of the components of the mixture. This is likely to be a very complex and expensive operation. Furthermore, the interpretation of measurements of a given chemical may be complicated by possible interactions with other chemicals in the mixture, which can alter the toxicokinetic behaviour of the compound in question. Suitable methods for characterizing exposure to mixtures of chemicals will require further research.

The methods for detecting mutagens and carcinogens have been developed largely for the analyses of food, although some have been applied to air and water. Their application to human tissues is more problematical because of bioconversion of the chemicals and the low concentrations present. Promising analytical approaches include immunoassay techniques which have been shown to be capable of detecting carcinogen metabolites in the urine of laboratory animals, but which have yet to be applied to human studies on a significant scale.

Methods for identifying covalent adducts formed by interaction between environmental chemicals and biological targets (such as DNA, RNA, and proteins) can in principle serve as sensitive dosimeters of exposure, especially since some adducts are excreted into the urine. As yet, however, the methodology for applying this approach to human specimens has been developed only to a limited degree. Further development and validation of these methods will be necessary before they can be used for monitoring human populations.

Several methods are available for obtaining indirect evidence of exposure to chemicals. These include measurements of changes in the activity of mixed function oxidase, as reflected in altered rates of elimination of aminopyrine, antipyrine, or other test substances. These alterations are so non-specific, however, that their practical usefulness as indices of exposure to mixtures remains to be determined.

Potentially useful methods for assessing exposure to genotoxic agents include cytogenetic analysis of blood lymphocytes and the evaluation of sperm motility and morphology. Cytogenetic and sperm abnormalities are, however, non-specific, and the relevant methods require further development and validation, particularly in relation to the effects of mixtures.

Additional methods for assessing exposure to genotoxic agents, alone or in mixture, make use of various other *in vitro* assays of human cells and/or body fluids. These include tests for the presence of mutagens in blood, urine, or faeces, for cytogenetic changes in blood lymphocytes and for the ability of lymphocytes to repair DNA damage *in vitro*. The ultimate usefulness of these methods remains to be determined.

1.2.2 Clinical and Laboratory Procedures

Clinical tests represent a group of methods for assessing the human health effects of exposure to single chemicals or mixtures. These tests include a broad spectrum of procedures, ranging from determination of the activity of some enzymes, to psychometric and behavioural analyses. In general, although these methods are not applicable to the monitoring of large populations, they may serve to screen defined populations of high-risk individuals: for example, individuals with congenitally subnormal acetylating capacity, with deficiency of glucose-6-phosphate dehydrogenase or plasma α_1-antitrypsin, or with specific HLA haplotypes. The identification of high-risk individuals may be helpful in monitoring exposed populations for potentially adverse effects, and in preventing the exposure of particularly sensitive persons within such populations.

1.2.3 Epidemiological Methods

Epidemiological methods play an essential role in assessing the human health effects of exposure to chemicals since, in the final analysis, human health effects

of a given chemical, or combination of chemicals, can be established conclusively only through epidemiological evidence. A variety of epidemiological methods are available, the relative merits of which vary depending on the circumstances; however, none of the methods are sensitive enough to detect small differences in the frequency of a given disease or abnormality. Moreover, the methods are able to record effects only after they have already occurred; in the case of chemically induced cancers, this means years or decades after the onset of exposure. Hence such methods are unable to provide warning far enough in advance to enable exposure to be prevented. Their limitations notwithstanding, existing epidemiological methods could be exploited more adequately if relevant data bases, records, and record-linkage systems were improved. Such methods would also be more effective if they were combined more consistently with complementary clinical and laboratory investigations.

1.2.4 Animal Assays

Methods for detailed analysis of the interactive effects of chemicals in mixtures and for elucidation of their toxicological mechanisms make use of comparative assays in experimental animals. For this purpose, various species (e.g. a mammal, bird, aquatic species, insect) are selected to represent a broad range of susceptibilities. Aquatic species have been particularly useful for studying the complex mixtures that are found in effluents discharged into lakes and rivers. Methods for evaluating in detail the potential human toxicity of individual substances customarily involve assays in rodents and, to a lesser extent, larger mammals (such as dogs and monkeys). Although specific protocols have been formulated, there has been little use of such methods for evaluating the effects of chemical mixtures. Hence their usefulness for this purpose is not established.

Since mammalian assays are costly and time-consuming, faster and more economical assays have been explored as screening tests, especially for genotoxicity and carcinogenicity. Such short-term tests, used in appropriate combinations, yield results consistent with those of rodent carcinogenicity assays for up to 80 % of the chemicals analysed to date. As yet, however, the methodology of such assays is still in an early stage of development. Comparable short-term tests for effects other than mutagenicity and carcinogenicity are less developed or not available.

Several extrapolation procedures are used to apply the results of animal assays to toxicity in human subjects. One method involves determining in experimental animals the potency of the chemical in question in relation to other, similar, chemicals and comparing this relation with the known potencies of the other chemicals in human subjects. An alternative procedure is the use of quantitative structure–activity relationships, especially those that permit extrapolation from data on one chemical to another of related structure, for example those within the same homologous series. Another method involves the use of theoretical models

for extrapolating (transposing) from animal species to man, and for extrapolating from observations under one set of exposure conditions to another. In general, models in current use are based on simplifying assumptions that remain to be validated. Their eventual refinement will depend on further insight into the mechanisms of the effects in question. Since these methods have had only a limited application to mixtures of chemicals, further research on such applications is needed.

1.3 METHODS FOR ASSESSING THE EFFECTS OF MIXTURES OF CHEMICALS ON NON-HUMAN BIOTA

Estimations of the exposure of non-human biota to complex mixtures of chemicals are not appreciably different from those for human individuals and populations. The same problems, limitations and qualifications apply, except for those related to occupational exposure and life-style which are unique to human exposures. The greatest similarities in estimating exposure of human and non-human biota occur when one examines what occurs within an organism, such as disruption of enzyme systems, tissue damage, and impairment of some physiological function. The chemical pathways likely to lead to exposure of both human and non-human organisms (e.g. chemical partitioning, transformation, and transport within the environment) may be quite similar in principle. However, in practice, since the world is highly urbanized, most human exposure is likely to occur at the workplace, at home, in automobiles, and in other places that can best be described as a man-made environment. Man-made environments tend to be more homogeneous and subject to less variability and regional differences than natural environments.

In the study of effects of mixtures of chemicals on non-human biota, there are literally thousands of species with variations in response among species sometimes exceeding an order of magnitude. In addition to the multiplicity of target species, one is also dealing with different end-points when considering individuals, populations, or ecosystems.

In studying the effects of chemicals in the environment, one is almost inevitably considering mixtures. Thus the decision is not whether or not to study mixtures, but when a detailed evaluation of chemical interactions in the environment is justified. Examples of major interaction are few and no definitive case of significant environmental damage caused by synergism has been reported. Whether this indicates a low frequency of such events or failure to detect them is a matter of debate. The detection of interactions of chemicals in the environment may come either from observation of an adverse effect in the environment or by prediction of possible interactions from knowledge of the mechanism of action of the components. At the moment, our knowledge of kinetics and mechanisms of action are largely inadequate for this type of prediction to be made. Studies of

chemical interactions will need to be broadened before this approach can be effectively used.

Toxicologists who estimate effects of chemical mixtures on both human and non-human biota must cope with responses at different levels of biological organization ranging from subcellular structures to ecosystems. At each higher level of biological organization, new questions are added that may not have been relevant at the lower levels. For example, predator-prey behaviour cannot be studied with a single species, nor can nutrient and energy transfer from one trophic level to another, yet both are important attributes of natural systems. Therefore, instead of focusing on a single species, the ecologist generally approaches complex problems from a systems analysis point of view. In this approach, first, the environmental system is defined by determining its boundaries in both time and space, and then the behaviour of the system is observed, including its natural variability. The next step is to determine the internal dynamics of the system and, finally, to identify the components within the system relevant to the observed behaviour, determine their linkage, and carry out experiments to show how they affect the overall system behaviour. Thus, this approach focuses on the larger system first, and through examination of the subunits affecting the most important processes it is then decided which of these units to use for testing. Since ecosystems vary quite markedly from one geographical region to another, the outcome of this approach is not likely to result in the same degree of standardization that may be possible when testing the toxicity of mixtures for evaluation of human health effects. The advantage of the systems analysis approach is that it permits a logical dissection of a complex system, always with reference to the overall behaviour of the system. It does not require a complete analysis of all components or all interactions. The main disadvantage is the difficulty of extrapolating from a part of the system to the whole. Major predictions made on a conceptual model should be verified in the field.

There are considerable difficulties in establishing ecosystem boundaries because there are no truly closed subsystems in the biosphere in which we live. All parts may and frequently do interact. Therefore, the isolation of a subsystem, although a necessary act in the process of hazard analysis, runs the risk that an important external variable may be overlooked. The bottom line is that it is highly improbable that ecologists will develop a 'white rat' system to represent a large set of environments. It is more likely that, when the testing at higher levels of organization reaches a stage of development enabling routine use, there will be a series of alternatives with perhaps a standardized approach rather than a standardized system.

Unfortunately, testing at levels of biological organization higher than a single species is still in the early stages of development, and most testing is done on single species and with single chemicals. From the vast array of species in the natural environment, a relatively few species have been selected that can be cultured in

laboratories and are thought to be representative in response. It does not appear from tests on these organisms that mixtures of chemicals pose any greater threats to organisms than a summation of the effects produced by the component chemicals. However, until these mixtures can be studied in complex systems and at higher levels of biological organization, it will not be possible to make this statement with assurance. Since the systems analysis approach has only begun to be used in the toxicity testing of non-human biota, it is unlikely that a final answer on the effects of mixtures of chemicals at higher levels of biological organization will quickly become available. Until then, one must hope that the extrapolations made from the relatively few test species of both terrestrial and aquatic systems are reasonably accurate, and that at environmental concentrations no significant, additional responses would occur at higher levels of biological organization than with single species.

Although synergism appears to be unlikely at the usual low concentrations of environmental pollution, there are good reasons for detailed studies of synergistic mixtures under certain circumstances. These include areas contaminated by multiple sources where significant adverse effects are documented, and situations where an effluent containing a complex mixture is known to have adverse environmental effects. In both cases, knowledge of component(s) which cause damage may allow us to undertake action short of an outright ban.

The basic dilemma is that the testing of mixtures inherently requires a large number of experiments to evaluate the interactions and relative contribution of the components, and thus tests have to be relatively simple; however, these tests give little information on the likely impact at the population level, let alone the ecosystem level. It is, therefore, important to follow multiple lines of evidence since specific tests can be expected to show some responses but not others. Some short-term tests, such as those on algae and daphnia, nevertheless cover several generations of these rapidly reproducing species. While many comparisons of acute toxicity have been made across many different phylogenetic orders, little comparison seems to have been made between the effects of chemicals on growth and reproduction of these simple organisms with those seen in higher species or in microcosms. Microcosms are a useful, and relatively realistic, method of measuring the effects of mixtures of chemicals on several different species. Both cost and increasing pressure against using higher animals for experimental work are likely to impose increasing dependence on tests with microorganisms. Field validation will then be important as a first step in confirming the significance of laboratory findings.

1.4 GENERAL CONCLUSIONS

(1) There is increasing evidence that many disease clusters in communities arise out of the complex interplay of more than one environmental causative agent (physical, chemical, or biological) and host factor. Such disease clusters and

episodes of pollution of the environment by chemicals tend to be accompanied by intense public concern which often pressures policy makers to reach decisions before experimental or epidemiological data can be collected and analysed. The currently available epidemiological methods have assisted in the identification of specific risk factors but have rarely permitted quantitative or even a semi-quantitative evaluation of the health effects resulting from exposure to mixtures of chemicals, and of interactions among chemical, physical, and biological agents.

(2) A variety of methods are available for assessing exposure to chemicals and their effects, based on different types of observations in the environment, in human subjects, and in experimental animals. The methods include: analytical procedures to measure chemicals in body cells, tissues, fluids and excreta; clinical and laboratory tests to detect the adverse effects of chemicals at various levels of biological organization; epidemiological methods to identify and characterize effects in exposed populations; and techniques for risk estimation and assessment based on extrapolation (transposition) of toxicological data pertaining to experimental animals.

(3) Many, if not most, of the methods available have been developed for estimating and assessing exposure to, and effects of, individual chemicals. The extent to which they are applicable to complex mixtures varies, depending on the method in question. An analytical method for identifying a specific chemical will not necessarily detect other chemicals in a mixture; the presence of other chemicals may alter the toxicokinetic behaviour and toxicological effects of a specific chemical in ways that are not apparent or predictable. The usefulness of many of the existing methods for estimating exposure to, and effects of, mixtures of chemicals is thus limited. In spite of their limitations, existing methods provide important approaches to the study of mixtures, which warrant further attention.

(4) Studies of mechanisms have concentrated heavily on the liver mixed function oxidase system. Predictions and experimental validation of chemical interactions have been largely confined to that system; beyond this there has been little prediction of chemical interactions from known mechanisms.

(5) The likelihood of significant synergism in nature appears to be low, and the rationale for studies of mixtures is more likely to be to minimize the impact of emissions and/or effluents from an industrial process by identifying active constituents for remedial action.

(6) The link between the bioassay test data frequently used as the basis for regulatory action and the actual environmental impact of chemical mixtures remains tenuous.

1.5 GENERAL RECOMMENDATIONS

(1) Further research into chemical interactions is central to the interpretation of studies on human health effects of mixtures of chemicals. Such research is needed

to indicate the extent to which the toxicokinetic behaviour and toxicological effects of one compound may be affected by the presence of other substances; quantitation is especially important.

(2) Analytical methods for detecting the covalent adducts that chemicals may form with blood proteins, cellular DNA, and other body constituents are potentially sensitive dosimeters of exposure to chemicals, including mixtures. Further research on the methods for measuring such adducts is strongly called for.

(3) Other biological indices of exposure to chemicals, such as changes in enzyme activity, cytogenetic alterations, perturbations in physiological functions, and the mutagenicity of body fluids, also deserve further study for their applicability in monitoring the exposure of populations to mixtures of chemicals.

(4) Additional research should be conducted to advance the methodology of bioassays for toxicological evaluation of chemical mixtures, including further development of short-term tests, whole animal long-term tests, and various combinations of such tests.

(5) Records, record-linkage systems, and data bases should be strengthened to facilitate epidemiological surveillance of exposed populations. Epidemiological studies should also be integrated more closely with, and reinforced by, complementary clinical and laboratory investigations as much as possible.

(6) Characterization of mechanisms underlying synergistic interactions should be broadened beyond the current emphasis on the liver to include other likely mechanisms of potent interactions.

(7) Links between animal and short-term bioassay data should be looked for and any predictions of comparative effects tested to improve our capacity to extrapolate from short-term tests to environmentally realistic conditions which almost invariably involve mixtures.

(8) The inadequacy of data on exposure of human and non-human biota both to single chemicals and to mixtures of chemicals is one of the main constraints in the estimation of dose–response relationships. Therefore, a separate study of methods for exposure estimation and assessment should be undertaken, including the use of modelling procedures.

1.6 REFERENCES

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