Methods for Assessing the Effects of Mixtures of Chemicals
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Biochemical Mechanisms of Combined Action

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ABSTRACT

Studies of complex mixtures can be approached by fractionation of the mixture followed by bioassay of the fractions or by building up the mixture from its components and testing various combinations. The first approach involves detailed analytical chemistry; the second is less demanding analytically but is likely to involve more bioassays. Detailed studies of mixtures can only be justified under special circumstances such as in areas contaminated from multiple sources when significant adverse effects are seen or when components of an effluent or mixture are causing problems.

A major goal is knowledge of interactions leading to synergistic reactions; but despite all the studies that have been carried out on mechanisms, the current approaches to the toxicology of mixtures are still largely empirical.

1 INTRODUCTION

Most of the studies of the effects of mixtures of chemicals have been carried out by treating the mixture as a single entity in that the mixture (industrial effluent, river water, crude oil, etc.) is put into the test system and the result observed. These studies are valuable since they give information on the effects that occur from a mixture at a certain concentration and thus predict the effect that can be expected, i.e. from a factory effluent. However, nothing is learned about the interactions of the various components of the mixtures on the target organisms and studies that treat mixtures as single entities will not be considered further here.

The two major approaches for elucidating the effects of individual chemicals in a mixture are by fractionating the original mixture and testing the fractions and comparing them with the effects of the original mixture, or by building up mixtures of specific chemicals to mimic the mixture. These approaches are often prohibitively expensive. Typically, a mixture would have to be analysed by such

techniques as gas chromatography/mass spectrometry (GC/MS), chemically fractionated, the individual fractions re-analysed and then tested on the target organism. The analytical chemical back-up for the second approach is not as important but the number of combinations of chemicals to be tested soon becomes impractical. Hence, from a practical viewpoint one is often forced back to treating mixtures as a single entity and thus treating factory effluent, or complex mixtures of isomers such as PCBs (polychlorinated biphenyls), as if they were single substances.

Nevertheless, there are situations and reasons where the detailed investigation of mixtures is worthwhile. These include the following:

(1) bodies of water contaminated by multiple sources where significant adverse effects are seen and when it is impossible to assign effects observed to any specific source;
(2) knowledge of which component(s) of an effluent or mixture of isomers is causing the problem may enable simpler clean-up procedures or reformulation to solve the problem rather than invoke a complete ban; and
(3) knowledge of interactions may enable synergistic reactions to be predicted so that the effects of most mixtures can be assumed to not cause problems greater than would have been expected from additivity, for example in predicting the load an ecosystem can take.

The methodologies needed to handle these situations are different with respect to problems involved; each will be considered under a separate heading.

2 KINETIC AND DYNAMIC PHASES OF INTERACTION

The interaction of chemical mixtures with an organism can be divided into two phases: kinetic (or pharmacokinetic), and dynamic (or pharmacodynamic) (Anderson and d'Apollonia, 1978; Ariens, 1972). In the pharmacokinetic phase there are the physiological interactions which affect the toxicants' compartmentalization, and metabolism which alters the concentration and the form in which the toxicant reaches the receptor sites. In the pharmacodynamic phase one is concerned with the affinity for, and interactions with, the target receptor, causing responses that lead to the final effect.

In real-life situations the picture can be more complex; in a food chain the pharmacokinetic phase may be repeated several times and the final chemical mixture to which the target organism is exposed can be quite different from the composition of the initial chemical input. Examples of this are the differential metabolism of PCB isomers and of the various hydrocarbons of crude oil. In both cases the final composition (at exposure) is very different from the initial (at release).

3 INVESTIGATIONS OF LARGE AREAS POLLUTED BY MANY SOURCES

The problem of tackling a large area polluted by many different chemicals from different sources can be divided into two main parts: (a) field studies such as studies of reproductive failure of the herring gull (*Larus argentatus*) in the Great Lakes of North America (Gilman *et al.*, 1977) or the ringed seal (*Pusa hispida*) in the Baltic (Helle *et al.*, 1976); and (b) laboratory studies such as effects of Rhine water on the rainbow trout (*Salmo gairdneri*) (Poels *et al.*, 1980). Consideration of the techniques is outside the scope of this review as essentially they involve investigation of effects in a specific area or they treat the mixture as a single entity.

The first phase of the problem of demonstrating which chemical(s) are responsible is the identification of what chemicals are actually present in what concentration in various parts of the ecosystem. This can be an analytical nightmare in itself. Some 800 pollutants have been recorded in the Great Lakes (IJC, 1983), although this enormous number is largely composed of PCB and toxaphene isomers and polynuclear aromatic hydrocarbons.

In the case of the herring gulls on the Great Lakes, attempts were made to identify the chemical(s) responsible for embryotoxicity by injection of pollutants back into gull eggs in a clean marine colony (Gilman *et al.*, 1978). Liposoluble pollutants were extracted from gull eggs collected in the Great Lakes. This material, as well as fractions of this material, were used in addition to synthetic organochlorine mixtures. However, no increase in embryonic or chick mortality was observed over the control group. Subsequent experiments injecting tetrachlorodioxin also failed to pinpoint the causative agent. Thus, these experiments, which were very time-consuming in the field studies and in the chemical back-up (analysis by GC/MS, extraction and purification of material for injection), failed to identify the causative agent(s). In the event, control measures under the Canada–US Great Lakes Water Quality Agreement and other actions that were occurring at that time (severe restriction of the use of most organochlorine pesticides, the withdrawal of most uses of PCBs) caused marked improvement in the reproductive success of fish-eating birds on the Great Lakes. In fact, this improvement, welcome though it is, confounded much of the studies since work was carried out while the problem and its cause were in fact disappearing.

In the Baltic, some 40% of the population of seals showed pathological changes in the uterus and markedly decreased reproductive potential. These findings had a significant, positive correlation with PCB levels. Some additional confirmation was obtained from experimental studies with another fish-eating mammal, the mink (*Mustela vision*), which showed reproductive failure with PCBs but not with DDT at environmental levels, although the pathological findings were not identical (Jensen *et al.*, 1977).

Detailed GC/MS work has revealed the presence of phenolic, hydroxylated and methyl sulphone derivatives of PCBs and DDE (Jansson *et al.*, 1975; Jensen

and Jansson, 1976). Since the levels of these compounds are positively correlated with those of their parent compounds, it is not possible to ascertain the relative importance of these compounds in causing the observed effects, especially since experimental studies on test organisms are not available.

While the analytical work in both of these studies has revealed the presence of a large number of anthropogenic substances, there is no proof that the key substances have in fact been identified. For example, the highly toxic tetrachlorodioxins were identified in the Great Lakes after the major parts of these studies had been completed (Norstrom *et al.*, 1982).

4 PHYSIOLOGICAL RESPONSES TO KNOWN COMPLEX MIXTURES

In a number of cases the pollutant mixture, although complex, can be defined, thus greatly decreasing the uncertainty since the boundaries of the required analytical work can be defined. Nevertheless, even here the caveat has to be added that background contamination, i.e. the essentially global nature of exposure to low levels of PCBs for example, has to be considered as a possible complication. The approaches that can be used are either fractionation of the original mixture or building the original mixture up from known compounds. Two specific examples are considered: the physiological response of the avian embryo when exposed to crude oil and the induction of mixed function oxidase in the quail by PCBs.

4.1 Physiological Responses of the Avian Embryo to Crude Oil

Crude oils have been shown to be embryotoxic to a variety of avian species when microlitre quantities are applied to the shells of their eggs (Albers, 1977; White *et al.*, 1979). Embryonic mortality, growth retardation, and teratogenicity are particularly marked during the first few days of embryonic development (Hoffman, 1978). Detailed studies of the major aliphatic hydrocarbons, aromatic hydrocarbons and standard reference crude oils showed that embryotoxicity is dependent on the aromatic hydrocarbon content, especially the polycyclic aromatics (Hoffman, 1979). Subsequently, it was shown that the addition of specific polycyclic aromatics to a synthetic petroleum hydrocarbon base of low embryotoxicity resulted in effects equivalent to that of the crude oil (Hoffman and Gay, 1981). Interestingly enough, the high molecular weight aromatic fraction was the one found to be biologically active in the retardation of growth and associated biochemical changes in the nesting gull (Peakall *et al.*, 1983).

4.2 Differential Response of Enzyme Induction to PCB Isomers

Bunyan and Page (1978) compared the effects of individual polychlorinated biphenyl isomers with those of commercial PCB mixtures. They examined the

residue levels and the degree of hepatic enzyme induction in the Japanese quail (*Coturnix coturnix*). They found that the residue levels varied greatly from isomer to isomer (from non-detectable to 68 ppm) and that large residue levels were generally associated with the degree of enzyme induction. The results make it clear that chlorinated biphenyl isomers cannot be considered as a group of compounds with identical or even similar effects on biological systems. This approach calls for the synthesis of the individual components of the mixture or the separation of the mixture into individual compounds; both of these approaches are frequently costly. However, from an environmental viewpoint the approach could have practical application. For example, it should be possible, based on Bunyan and Page's studies, to devise a mixture of PCB isomers which would have low biological stability and effect (at least in the system under investigation); if this mixture had suitable electrical properties along with thermal stability and could be commercially produced, then it could be an environmentally acceptable alternative to PCBs.

5 PREDICTION AND TESTING FOR SYNERGISM

One important rationale for examining the effects of mixtures of chemicals is the possibility that synergistic reactions will cause problems that are more serious than would be predicted from the toxicity of the individual compounds alone. The methodology for the examination of binary mixtures and the evaluation of the effect–concentration curves (usually displayed as mortality probits against log concentration) is well advanced. The mathematical treatment goes back to the work of Bliss (1939) and was elaborated in a series of papers by Plackett and Hewlett (e.g. Plackett and Hewlett, 1952). These workers proposed the following classification:

	Similar joint action	Dissimilar joint action
Interaction absent	Simple similar action	Independent action
Interaction present	Complex similar action	Dependent action

Anderson and Weber (1975) have proposed the term concentration–addition rather than similar joint action and response addition rather than independent joint action. Applying these classifications to mixtures of more than two chemicals can be difficult as the joint action of the different pairs can fall into different classes. Thus, the mathematical treatment of joint toxicity of multi-chemical mixtures is difficult (Könemann, 1981) but the greatest problem is the astronomical number of combinations that are possible.

5.1 Rationale for Studies of Mixtures

Since the number of possible combinations of pollutants is so large, some rationale for the priority of testing has to be developed. This could include:

(1) tests of combinations of chemicals that are to be used in the environment;
(2) tests when additional compound(s) are added to a system known to be already contaminated with other compound(s); or
(3) tests of combinations when there is an *a priori* reason to suspect that interaction may occur.

5.1.1 Pesticides

One of the major classes of toxicants that reaches the environment in combination is that of pesticides. Macek (1975) evaluated the acute toxicity to bluegills (*Lepomis macrochirus*) of 29 two-pesticide combinations. The most strongly synergic was the combination of the two organophosphates malathion and parathion; the combination of organochlorines or organophosphates with organochlorines was generally additive or less than additive. Other compounds frequently found together are the heavy metals. A great deal of work has been done on combinations of heavy metals, especially on fish. A comprehensive review of this work has been produced by FAO (1980). Uptakes of heavy metal combinations are complex; for example, the uptake of lead and cadmium is inhibited by low concentrations of copper but enhanced by higher concentrations.

Since the occurrence of organochlorine compounds (especially DDE and PCBs) is essentially global in nature, the evaluation of other potential toxicants has to be viewed against this background. Ludke (1977) examined the effects of parathion on cholinesterase activity and mortality in quail. He found enhanced decreases of plasma cholinesterase and mortality in quail pretreated with DDE and then challenged with parathion. The rate of conversion of parathion to paraoxon did not appear to have been affected and it was considered that a decreased rate of paraoxon detoxification was the most likely cause. Similar interactions were found between mercury and parathion (Dieter and Ludke, 1975). In contrast, no effect was found on the combined action of DDE and an organophosphate (acephate) in behavioural studies on the kestrel (*Falco sparverius*) (Rudolph *et al.*, 1984).

5.2.2 Detergents

These compounds constitute a widespread class of pollutants and have been studied for their possible interactions with other toxicants. Since the function of detergents is to lower the surface tension of water, it is a reasonable hypothesis that this could cause an increase in cellular permeability and thus increase the penetration of the other toxicant. More than additive effects have been observed.

Solon and Nair (1970), for example, found a synergistic effect between linear alkylbenzene sulphonate and parathion in their studies of toxicity to the fathead minnow (*Pimephales promelas*). Calamari and Marchetti (1973) examined the toxicity of mixtures of metals in relation to two anionic and non-ionic surfactants. They concluded that the physical effect caused by the surfactants was definitely much less important than the chemical effect each compound exerts independent of its surfactant properties. From a practical viewpoint, these authors conclude that the excesses over additive responses seen with toxicants and surfactants are small compared with the safety factors used in setting water quality standards.

6 PREDICTION OF SYNERGISM

The underlying mechanism of synergism has been reviewed by Wilkinson (1968). He points out that detoxification usually occurs in two major steps: modification of the molecule to render it more polar, followed by conjugation so that the foreign material can be excreted. Synergism of insecticides usually occurs by inhibiting the enzymes responsible for the detoxification of the insecticide (Metcalf, 1967). In some specific cases, such as sesamex, which has been used commercially to enhance the toxicity of pesticides, there is a good underlying biochemical theory for synergism. Structure–activity relationships for the blockage of oxidative dealkylation in insects has been developed for methylenedioxyphenyl (1,3-benzodioxole) (MDP) compounds (Hodgson and Philpot, 1974). In this case, the synergistic activity of MDP compounds results from a stable inactive complex with cytochrome P-450. These studies, while of fundamental mechanistic importance, do not have widespread application to the prediction of synergism in environmental situations.

Inducers of mixed function oxidase (MFO) activity are common (organochlorines, polynuclear aromatics) and in this case the half-life of one pollutant can be decreased if the organism has been pre-exposed to an inducer (Street, 1968). Nevertheless, the ability to predict interactions under realistic conditions remains very limited.

7 CONCLUSION

A recent panel (Stich *et al.*, 1982) concluded, 'Simply stated, our current understanding of the fundamental scientific principles of chemical interactions and methodology is insufficiently developed to permit the required assessments.' Two approaches can be envisioned: tests that can be carried out rapidly enough so that it is feasible to test a large number of environmentally relevant mixtures, and secondly, prediction of effects based on some physicochemical property of the chemicals involved. Examples of the former are the use of tissue culture, rather than inhalation studies of intact animals, to assess the effect of airborne

pollutants (Rasmussen and Crocker, 1981) and the use of the Ames test and sister chromatid exchange integrated with analytical chemical methods to track down mutagenic substances in polluted waters (Poels *et al.*, 1982).

An example of the second approach is an attempt to relate the induction of MFO systems to the strength of the carbon–hydrogen bond of the xenobiotic involved. The hypothesis is that the response of the MFO system to a combined dose is the induction of an isozyme corresponding to the component in the mixture with the strongest carbon–hydrogen bond (Hollebone and Brounlee, 1986).

Nevertheless, one has to confess that the amount of progress made since 1968 has not been great. In that year Brown (1968) used a simple empirical approach to calculate the toxicity of mixtures of five components with widely varying toxicities and found that the summation of the toxicities of the individual compounds was within the 95 % confidence limit of the observed toxicity of the mixture.

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