SESSION VI

TOXIC EFFECTS PRODUCED IN VERTEBRATES BY ANTICHOLINESTERASES

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The Toxicity of Organophosphorus Compounds to Mammals

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The acute toxicity of most of the commonly used organophosphorus insecticides is essentially the same. A few compounds with low toxicity, such as malathion, have been developed but further efforts in that direction are needed. Most of the organophosphorus insecticides exert a generalized cholinergic action by inhibiting central and peripheral cholinesterases. The phosphoramides are an exception in that they do not gain access to the cholinesterase of the central nervous system in vivo *and consequently atropine is a more effective antidote for them than for organophosphorus compounds. Young animals are more susceptible to the organophosphorus compounds than are adults. Enzyme-inducing agents decrease the toxicity of the phosphorothioates and phosphorodithioates. All organophosphorus insecticides can inhibit esterases that catalyse the detoxification of some insecticides of this class and ester-type drugs.*

The toxicology of the organophosphorus insecticides has received a tremendous amount of attention during the past 25 years because of the established value and widespread use of these compounds as agricultural insecticides. The use of organophosphorus compounds as insecticides has prompted extensive investigation of their toxicity to various laboratory animals as a means of evaluating the possible hazards to the health of those engaged in their manufacture and use and those who may consume food containing small residues of these agents.

Any consideration of the available data on the toxicity of organophosphorus insecticides must take into account the fact that studies of this type are generally conducted in connexion with some aspect of their practical use, because toxicological studies are costly and time consuming. As a result, each group of investigators conducts toxicity tests in which the types of experiment and the procedures used are selected with primary consideration being given to the practical problems at hand. Thus, toxicity studies are frequently carried out on technical materials of the same purity as those used for insecticidal purposes rather than on highly purified materials. The solvents that are used to dissolve the compounds for experimental studies or that are present in various formulations sometimes influence the toxicity of the organophosphorus compounds. Nevertheless, sufficient toxicity data are available to permit relatively accurate comparisons of the toxicity of various members of the group.

At present, the toxicological evaluation of organophosphorus insecticides usually consists of a series of experiments that include the measurement of (*a*) acute toxicity by several routes of administration, (*b*) subacute toxicity by repeated injection or by feeding the compounds, and (*c*) chronic toxicity by feeding various low levels of the compounds in the diet for long periods of time. With this particular group of compounds measurement of the anticholinesterase action has become an established part of toxicity evaluations. This paper discusses the toxicity of a number of organophosphorus insecticides and considers their mode of action and the way in which they are metabolized, in so far as these factors are related to and govern the toxicity of these chemical agents.

Acute toxicity is the problem of greatest importance for the organophosphorus insecticides. Acute intoxication by every member of this group always results in at least some symptoms characteristic of stimulation of the parasympathetic nervous system (muscarinic effects). These symptoms, which consist of bronchoconstriction, sweating, salivation and other increased glandular secretions, anorexia, nausea, abdominal cramps, vomiting, diarrhoea, invol-

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untary defaecation, and increased urination, are of paramount importance because all organophosphorus insecticides produce some or all of the muscarinic actions of acetylcholine. The actions of organophosphorus compounds on skeletal muscle (nicotinic effects) include muscular twitching, muscular fasciculation, increased liability to fatigue, and muscular weakness (which also involves the muscles of the respiratory system). Most of the organophosphorus compounds are able to gain access to the central nervous system, where their action results in anxiety, restlessness, impairment of memory, speech defects, convulsions, and coma. The signs and symptoms produced by anticholinesterase agents in different mammalian species are essentially similar quantitatively, suggesting similar tissue distribution patterns. However, the doses required to elicit toxic effects in different species vary considerably, probably owing largely to differences in the rate and extent of detoxification of the compounds.

PYROPHOSPHORIC ACID DERIVATIVES

In the early studies on the toxicology of organophosphorus insecticides a great deal of attention was given to the derivatives of pyrophosphoric acid and particularly to tetraethyl pyrophosphate (TEPP). Interest in this compound led to the investigation of other pyrophosphates containing various alkyl groups (DuBois & Coon, 1952). Table 1 shows the intraperitoneal toxicity of five derivatives of pyrophosphoric acid for mice and their anticholinesterase activity *in vitro*. The results of these studies demonstrated the influence of various alkyl groups on the toxicity and anticholinesterase activity of organophosphorus compounds and the correlation between *in vitro* anticholinesterase activity and toxicity. Since the toxicity of pyrophosphates can be varied over a considerable range by appropriate alkyl substitution, and since the hydrolysis products are simple nontoxic derivatives of phosphoric acid, it seems that these organophosphorus compounds might be worthy of further consideration in efforts to develop insecticides with low persistence and nontoxic degradation products.

Table 1

Toxicity and anticholinesterase action of alkyl pyrophosphates

Compound	Intraperitoneal LD_{50} (mg/kg)	I_{50} for ChE *in vitro* (M)
tetramethyl pyrophosphate	1.7	1.8×10^{-8}
tetraethyl pyrophosphate	0.85	4.0×10^{-9}
diethyl dimethyl pyrophosphate (asymmetrical)	1.1	8.0×10^{-9}
diisopropyl dimethyl pyrophosphate (asymmetrical)	2.5	2.0×10^{-7}
tetraisopropyl pyrophosphate	16.0	1.4×10^{-6}

Schradan

Further studies of pyrophosphates in the early 1950s led to the finding that metabolic changes in the parent molecule can result in the transformation of an inactive compound into an anticholinesterase agent. The first observation along this line was made with octamethyl pyrophosphoramide (schradan), the analogue of tetraethyl pyrophosphate in which dimethylamino groups replace the ethoxy groups of TEPP. The toxicity of this compound is not strikingly different from that of the alkoxy pyrophosphates, since the LD_{50} values for several species by the parenteral route are 5–20 mg/kg. However, the replacement of alkoxy groups with amide groups resulted in a marked difference in the inherent biological activity of the compound and in its site of action. The ineffectiveness of schradan as an inhibitor of cholinesterase *in vitro*, in contrast to its strong anticholinesterase action *in vivo* (DuBois et al., 1950), is well known. The early demonstration that schradan is converted by an oxidative reaction in the liver to a metabolite with strong anticholinesterase activity (DuBois et al., 1950; Gardiner & Kilby, 1950) is also well known. It is unnecessary to describe the details of experiments on the oxidative activation of phosphoramides by the enzyme system that is now known to be located in the microsomal fraction of the liver. Knowledge that schradan must undergo biochemical transformation to exert an anticholinesterase action provided the first clear evidence that the potency of organophosphorus insecticides as inhibitors of cholinesterase cannot always be ascertained by conducting *in vitro* tests.

The other unique property of schradan that has not been intentionally exploited in the development of new insecticides is its selective action. Schradan produces all the muscarinic effects of organophosphorus insecticides and also the typical stimulant action on skeletal muscle, but it has no action on

Table 2

Selective anticholinesterase action of phosphoramides

Compound	Dose [a] (mg/kg)	Maximum inhibition of ChE (%)	
		Brain	Submaxillary gland
octamethyl pyrophosphoramide	5.0	0	88
diethyl bisdimethyl pyrophosphordiamide (symmetrical)	6.25	3	82
diethyl bisdimethyl pyrophosphordiamide (asymmetrical)	1.95	5	78
tetramethyl phosphorodiamidic fluoride	3.1	25	85

[a] Each dose represents 5/8 of the acute intraperitoneal LD_{50}.

the central nervous system. Small doses (5 mg/kg) given intraperitoneally cause marked inhibition of the cholinesterase of peripheral tissues but no inhibition of brain cholinesterase, which is consistent with the absence of signs caused by central nervous system stimulation. Further studies (DuBois et al., 1953) have demonstrated that an amide linkage in other pyrophosphates as well as in phosphorothioates also imparts a selective peripheral action. Table 2 gives some examples of organophosphorus compounds that exert a selective action on the cholinesterase of peripheral tissues. From the standpoint of mammalian toxicology the advantage of a selective peripheral action is that atropine is much more effective than it is in treating poisoning by compounds that also inhibit the cholinesterase activity of the brain.

PHOSPHOROTHIOATES

Of all the organophosphorus compounds, the phosphorothioates and phosphorodithioates have received by far the greatest amount of attention from toxicologists. The widespread use of these agents and the continued development of new compounds in these classes have served to maintain interest in their toxicology. In addition, a number of interesting features of the toxicity of these compounds have attracted the attention of many investigators interested in the reasons for age and species differences in susceptibility, especially in relation to the metabolism of the compounds. It can probably be safely stated that more is known about the details of the factors governing the toxicity of organophosphorus insecticides than is true for any other group of insecticides. However, efforts to utilize this information for the development of new agents have been somewhat limited.

It is probable that experience with parathion, the first member of this series to be widely used as an insecticide, served to indicate that organophosphorus compounds with high acute toxicity could be employed without large numbers of accidental poisoning incidents. On the other hand, the incidence of acute poisoning by parathion and other organophosphorus compounds with similar toxicity has been high enough to demonstrate that greater efforts should be made to replace the most toxic compounds of this class. Complete data on the toxicity of many phosphorothioates and phosphorodithioates have been published in many original articles and in reviews. Figures for the acute oral toxicity of a few common compounds of these classes are presented in Table 3.

The acute toxicity of the common phosphorothioates and phosphorodithioates is well known and only brief comments on certain aspects of this subject need be made. All these compounds show essentially the same pattern of toxicity, although their oxygen analogues show appreciable differences in potency as cholinesterase inhibitors and there are differences in the rate and type of metabolic degradation. The sex difference in susceptibility is largely confined to rats and is the result of a higher rate of metabolism catalysed by oxidative microsomal enzymes. The toxicity of all agents of this class when administered by the intraperitoneal route is approximately twice their toxicity when administered orally. However, members of this group show greater variation in toxicity when administered dermally than they do when administered

Table 3
Acute oral toxicity of several organophosphorus insecticides for male and female rats

Compound	LD_{50} (mg/kg) for:	
	females	males
parathion	4.0	7.0
parathion-methyl	4.5	9.7
EPN †	7.0	32.0
demeton	2.5	6.0
azinphos-methyl	10.0	18.0
fenthion	310.0	190.0
malathion	1 000.0	1 375.0

by other routes, and the overall structure of a compound is apparently more important in determining absorption by the dermal route than is the case for other routes.

Species differences in the toxicity of organophosphorus compounds for the common experimental animals are generally small. Age differences in susceptibility have not been routinely investigated. However, Brodeur & DuBois (1963) have compared the toxicity of 15 organophosphorus insecticides for weanling and adult male rats, and some of the results are given in Table 4.

Table 4
Acute toxicity of organophosphorus insecticides for weanling and adult male rats

Compound	Intraperitoneal LD_{50} (mg/kg) for:	
	weanlings	adults
parathion	1.5	3.6
parathion-methyl	3.5	5.8
EPN †	8.0	33.0
demeton	2.5	3.8
carbophenothion	5.4	9.4
ethion	100.0	128.0
azinphos-methyl	3.4	4.9
malathion	340.0	750.0
schradan	49.0	10.0
dioxathion	49.0	94.0

Weanling male rats (23 days old) are more susceptible to most of the phosphorothioates and phosphorodithioates than are adult rats, because the detoxification enzymes in the former have not yet developed to the adult level. The degree of age difference varies from one compound to another, and probably depends on the extent to which a given compound is detoxified by means of oxidation processes catalysed by microsomal enzymes. The detoxification of phosphorothioates and phosphorodithioates seems to be more dependent upon microsomal oxidation than that of the phosphates and phosphonates. It was clear from this study that age differences in susceptibility must be considered separately for each organophosphorus compound. It appears that conversion to the oxygen analogues requires only a small amount of the total microsomal oxidase activity in adult liver. Thus, the rate of detoxification would tend to have a marked influence on the toxicity of the compounds. Further studies should be carried out on young animals of various species.

INTERACTIONS WITH OTHER CHEMICALS

One important aspect of the toxicity of organophosphorus insecticides that has received relatively little attention is the increase or decrease in toxicity that may result from interactions with other chemicals or drugs to which the subject may be exposed simultaneously. The use of toxicity tests as a general procedure for detecting such interactions would not be practicable, owing to the numerous combinations of chemicals that would have to be tested. A more practical procedure is to consider the biochemical mechanisms that might be responsible for the increases and decreases in the toxicity of organophosphorus insecticides resulting from interaction with other chemical agents.

Enzyme induction

One interaction mechanism of obvious importance for the organophosphorus insecticides is interference by another chemical agent with the activity of the hepatic microsomal enzymes that activate and degrade these insecticides. It would be impractical to test the effects of enzyme-inducing agents in combination with each of the organophosphorus insecticides. For this reason DuBois & Kinoshita (1968) attempted to develop a procedure that could be employed generally to ascertain whether an enzyme-inducing agent affects the toxicity of any

Table 5
Influence of phenobarbital treatment on the acute toxicity of organophosphorus insecticides for rats and mice

Compound	LD_{50} (mg/kg) for:			
	rats		mice	
	control	phenobarbital-treated	control	phenobarbital-treated
parathion	2.45	7.3	8.1	14.2
parathion-methyl	7.0	8.0	9.3	14.3
EPN †	7.3	75.0	23.7	64.4
demeton	2.1	16.9	6.7	16.3
carbophenothion	10.1	70.5	27.0	44.0
ethion	25.9	302.6	34.5	40.8
azinphos-methyl	8.7	11.4	4.0	4.9
malathion	619.4	949.9	193.0	234.0
schradan	28.7	14.5	9.6	9.9
dioxathion	17.2	118.7	33.2	87.0

organophosphorus insecticide. The procedure used in these experiments consisted of the daily intraperitoneal administration of 50 mg of phenobarbital sodium per kg of body weight for 5 days. On the day following the fifth treatment with this broad-spectrum enzyme-inducing agent the acute toxicity of a number of organophosphorus insecticides was measured, and some of the results are given in Table 5.

The results of this study demonstrated that the treatment of rats and mice with phenobarbital for a period long enough to cause marked induction of hepatic microsomal enzymes decreases the acute toxicity for them of several common organophosphorus insecticides. A marked decrease in the toxicity of some compounds, including disulfoton, dioxathion, ethion, carbophenothion, and EPN † was observed in the phenobarbital-treated animals. Smaller decreases in the toxicity of some other compounds were noted, but the significant finding was that the induction of hepatic microsomal enzymes did not increase the toxicity of any of the compounds except schradan, which is activated but not detoxified by hepatic microsomal enzymes. Since phenobarbital treatment decreased the toxicity of most of the compounds it is apparent that the rate of microsomal detoxification is more responsive to enzyme-inducing agents than is the activation reaction.

† Names against which this symbol appears are identified in the Glossary on pages 445-446.

The demonstration that exposure to an enzyme-inducing agent does not increase the toxicity of organophosphorus insecticides is of considerable importance with respect to the combined toxicity of such insecticides and other chemicals. Any chemical agent capable of producing enzyme induction would be expected to influence the toxicity of organophosphorus insecticides in the same way as phenobarbital, or to a smaller extent if it induced fewer microsomal enzymes. From the mechanistic point of view, therefore, it is not necessary to test the effects of all potential enzyme-inducing agents to ascertain their possible effects on the toxicity of organophosphorus insecticides.

Many pesticides do not fall within a chemical class such as the organophosphorus compounds or the chlorinated hydrocarbons, and the possibility of interactions between such agents and other pesticides and drugs should also be studied. Measurement of the toxicity of these agents in combination with each important drug or pesticide is the most certain method of detecting potentiation or antagonism, but practical considerations limit its use. Since the alteration of detoxification enzymes plays a prominent role in causing interactions, our laboratory is attempting to elucidate the structural features of chemical agents that cause such alterations. We have developed a procedure for detecting the ability of various chemical agents to induce or inhibit

hepatic microsomal detoxification enzymes. An essential requirement for such a procedure is the availability of quantitative assay methods. Previous studies in this laboratory (Kinoshita et al., 1966) have resulted in the development of quantitative procedures for measuring the levels of oxidative microsomal enzymes. Our procedure for detecting the induction of enzymes by pesticides and other chemicals consists of two types of experiment. Initially, the acute toxicity of the pesticide is determined in mice and rats. Groups of 4 or 5 animals of each species are then treated daily for 5 days with 1/5 or 1/10 of the acute LD_{50}, and microsomal enzyme activity is measured on the sixth day. If enzyme induction does not occur under these conditions, further studies are considered to be unnecessary. When enzyme induction is observed, it is considered essential to carry out further studies to determine whether the effect occurs at a practical dose level. With pesticidal chemicals this is done by feeding various dietary levels in the vicinity of the established or proposed tolerance levels to rats under essentially the same experimental conditions as are used in the conventional subacute and chronic toxicity tests.

The procedure described above was applied to a number of pesticidal chemicals, few of which were observed to cause enzyme induction, and it was concluded that these agents would not alter the toxicity of other chemicals by changing their rates of metabolism. Occasionally, however, new classes of inducing agents are found; for example, Kinoshita & DuBois (1970) discovered that the substituted-urea herbicides produce this effect. When information of this type is available for a number of classes of insecticides, drugs, and other chemicals it should be possible to utilize it to predict whether or not interactions would occur as a result of changes in levels of microsomal enzymes, thus avoiding the necessity for conducting large numbers of toxicity tests.

Esterase inhibition

Another type of interaction for which a biochemical approach is now preferable to toxicity tests is the potentiation of the toxicity of various esters as a result of the inhibition of esterases (carboxylic ester hydrolases, B-esterases) by organophosphorus insecticides. Since Frawley et al. (1957) discovered the ability of EPN † to potentiate the toxicity of malathion, the toxicity of each new anticholinesterase agent under development as an insecticide has been measured in combination with each agent of the same class for which tolerance levels have been established. The increase in the number of anticholinesterase insecticides has resulted in a great increase in the number of toxicity measurements that must be made to ascertain whether any combinations cause potentiation. Furthermore, it has become apparent that the simultaneous administration of two compounds may not reveal the capability of one of them to potentiate the toxicity of the other. From the point of view of the mechanism involved, it is more logical to study the combined action of organophosphorus insecticides by measuring their potency as inhibitors of esterases than to measure the toxicity of every possible combination of compounds. It has become apparent from biochemical studies of the mechanism of potentiation that only one implication of esterase inhibition has been considered during the past 10 years—the possibility that one organophosphorus insecticide will potentiate the toxicity of another by inhibiting its detoxification. However, any compound, such as EPN,† that can inhibit esterases would be expected to inhibit not only the detoxification of another organophosphorus insecticide but also the hydrolytic detoxification of any drug or other type of chemical agent that is normally detoxified by esterases. It follows, therefore, that the procedure of testing the toxicity of different combinations of organophosphorus insecticides is too restricted, even if it involves a large number of tests, and to us it seemed more logical to devise quantitative methods of measuring esterase inhibition for determining the ability of these insecticides to potentiate the toxicity of other esters.

Recent studies in our laboratory (DuBois et al., 1968) resulted in the development of a procedure for measuring the potency of these insecticides as esterase inhibitors using diethyl succinate and tributyrin as substrates for liver and serum esterases. Different levels of a number of organophosphorus insecticides were fed in the diet for several weeks and assays were conducted periodically. It was found that, at a given level, maximum inhibition occurred when the diet was fed for only 1 week. This information was then applied to a study of 18 organophosphorus insecticides, in which different levels were fed in the diet for 1 week and the degree of inhibition of carboxylic ester hydrolase and cholinesterase activity. The dietary levels of each compound that would cause 50% inhibition of the enzyme activity were calculated from the results obtained.

Table 6

Dietary levels of organophosphorus insecticides that produce 50 % inhibition of hydrolysis of tributyrin and diethyl succinate by rat liver and serum

Compound	Dietary level (ppm) for 50 % inhibition of hydrolysis of:			
	diethyl succinate		tributyrin	
	liver	serum	liver	serum
parathion	4.0	6.7	1.5	7.2
parathion-methyl	8.8	> 25.0	2.5	> 25.0
EPN †	4.5	7.5	3.9	8.5
demeton	2.5	12.3	0.7	12.0
dioxathion	4.8	16.0	2.2	14.0
ethion	12.0	108.0	13.0	> 25.0
coumaphos	4.7	14.5	1.8	9.5
fenchlorphos	21.0	38.0	8.0	37.0
Folex †	1.7	2.6	1.6	5.0

Some of the results of these measurements are presented in Tables 6 and 7.

The results showed that all the organophosphorus insecticides inhibit the hydrolysis of diethyl succinate and tributyrin by liver and serum. Comparisons indicated that there is no consistent relationship between the potency of the compounds as inhibitors of carboxylic ester hydrolases and their potency as cholinesterase inhibitors. All the insecticides inhibited one or both of the carboxylic ester hydrolases at lower dietary levels than were necessary to produce an equivalent degree of cholinesterase inhibition. Differences exceeding 10-fold were observed between the inhibition of cholinesterase and that of carboxylic ester hydrolases produced by several insecticides, including EPN,† dioxathion, ethion, coumaphos, fenchlorphos, and Folex.† If information on dietary levels that inhibit esterase activity were to be used in establishing tolerances for organophosphorus insecticides, adherence to such tolerances would insure that interactions through this mechanism would not occur.

Table 7

Dietary levels of organophosphorus insecticides that produce 50 % inhibition of cholinesterase activity of the tissues of rats

Compound	Dietary level (ppm) for 50 % inhibition of ChE in:		
	brain	liver	serum
parathion	12.5	17.0	8.4
parathion-methyl	14.5	25.0	23.0
EPN †	40.0	> 100.0	125.0
demeton	5.4	15.0	5.0
dioxathion	54.0	48.0	27.0
ethion	108.0	70.0	60.0
coumaphos	80.0	50.0	25.0
fenchlorphos	280.0	90.0	90.0
Folex †	130.0	50.0	52.0

CONCLUSIONS

The toxicity of the organophosphorus insecticides has been extensively studied over the past 25 years, but—apart from a few new compounds—the amount of information on the subject has not increased appreciably since it was reviewed by DuBois (1963). The organophosphorus insecticides differ quantitatively in their toxicity for mammals, but most of the widely used ones are highly toxic. The pharmacological effects of different compounds are essentially the same in that they inhibit cholinesterase of the central and peripheral nervous systems and thus produce generalized cholinergic effects. However, octamethyl pyrophosphoramide and related compounds having phosphoramide linkages form an exception: these agents do not inhibit brain cholinesterase *in vivo* because they are unable to gain access to brain cholinesterase. Their selective action on peripheral tissues makes atropine a more effective antidote for them than for compounds that affect the central nervous system. This factor, together with the lack of biological activity of their hydrolysis products, may render pyrophosphates worthy of consideration for more extensive development as insecticides.

The phosphorothioates and phosphorodithioates are still the most important organophosphorus insecticides. These agents, of which parathion may be considered to be the parent compound, usually have high toxicity for mammals and exert a generalized cholinergic action. Only malathion, fenchlorphos, and a few other compounds have low toxicity for mammals. It seems worth while to make further efforts to develop organophosphorus compounds with low toxicity for mammals, especially if they could be used generally as substitutes for the chlorinated hydrocarbons for non-agricultural uses.

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DISCUSSION

HOLLINGWORTH: Would Dr DuBois comment on the suggestion that we concentrate too much of our attention on the liver as a metabolic tissue in mammals? This organ is highly active in both toxifying and detoxifying phosphorothioates, but I recall that some years ago Dr Gage reported that parathion is just as toxic to hepatectomized rats as to those with a liver. We know that many other tissues are also able to activate phosphorothioates. What is the relative importance of these other tissues compared with that of the liver, how does this change with age and sex, and what is the effect of dosage with inducing agents such as phenobarbital?

DUBOIS: Quantitative measurements of the rates of activation and degradation of phosphorothioates in tissues other than the liver have been attempted. In the rat, the liver is by far the most active organ for both processes, although the contribution of other tissues should be studied further. It is clear from studies that have been carried out on liver with a variety of phosphorothioates that we should not generalize on the basis of work on one compound, such as parathion. To obtain the answers to the questions asked by Dr Hollingworth, we shall have to consider all possible sites of activation and detoxification for every important compound.