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Biochemical Mechanisms In The Toxicity Of Some Vinyl Organophosphorus Compounds

Antonio Caste Morello

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BIOCHEMICAL MECHANISMS IN THE TOXICITY OF SOME VINYL ORGANOPHOSPHORUS COMPOUNDS

by

Antonio Morello Caste

Department of Biochemistry and

Research Institute

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Finally, our appreciation is conveyed to the Canada Department of Agriculture and the Allied Chemical Co., whose financial assistance made this investigation possible.

TO ANGEINETTA

The singer, not the song

CONTENTS

ABBREVIATIONS

DDT	1, 1, 1-trichloro-2, 2-di(4-chlorophenyl) ethane
DFP	di-isopropyl phosphorofluoridate
Dimephox	tetramethyl fluorophosphorodiamidate
Schradan	bix-N-dimethyl phosphorodiamidic anhydride
Parathion	ethyl parathion; diethyl, p-nitrophenyl phosphorothionate
Sarin	O-isopropyl-methyl phosphorofluoridate
Tabun	O-ethyl-N-dimethyl cianophosphoramidate
Phorate	O, O-diethyl, S-(ethyl-thiomethyl) phosphorodithioate
Bidrin	3-hydroxy-N, N-dimethyl-cis-crotonamide dimethyl phosphate
2-PAM	pyridine-2-aldoxime methiodide
bis 4-PAM	1, 3-bis(N-pyridinium-4-aldoxime) propane
Demeton	Systox, mixture of O, O-diethyl, O-ethyl-2-mercaptoethyl phosphorothionate and the thiol isomer O, O-diethyl, S-ethyl-2-mercaptoethyl phosphorothiolate
Amiton	O, O-diethyl-S-2-diethyl aminoethyl phosphorothiolate
Phosdrin	mevinphos, dimethyl, 1-carbomethoxy-1-propen-2-yl phosphate
Bomyl	dimethyl-1, 3-dicarbomethoxy-1-propen-2-yl phosphate
Desmethyl Phosdrin	monomethyl-1-carbomethoxy-1-propen-2-yl phosphate
Thiono Phosdrin	dimethyl, 1-carbomethoxy-1-propen-2-yl phosphorothionate

Thiono Bomyl	dimethyl, 1, 3-dicarbomethoxy-1-propen-2-yl phosphorothionate
TOCP	tri-o-cresyl phosphate, tri-o-tolyl phosphate
Dipterex	dimethyl-2, 2, 2, -trichloro-1-hydroxyethyl phosphonate
Malaoxon	dimethyl-S-1, 2-dicarbethoxyethyl phosphorothiolate
Malathion	dimethyl-S-1, 2-dicarbethoxyethyl phosphorodithioate
Methyl-parathion	dimethyl-p-nitrophenyl phosphorothionate
Ethyl-parathion	diethyl-p-nitrophenyl phosphorothionate
Sumithion	dimethyl (4-nitro-m-tolyl)-phosphorothionate

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SUMMARY

The geometrical isomers of mevinphos, dimethyl-1-carbomethoxy-1-propen-2-yl phosphate (Phosdrin^R) and of dimethyl-1,3-dicarbomethoxy-1-propen-2-yl phosphate (Bomyl^R) as well as their respective sulfuranalogs (thiono Phosdrin and thiono Bomyl) have been prepared and characterized. Their toxicities to the housefly and the mouse were determined.

The inhibition of bovine red cell, mouse brain, and fly head cholinesterase by Phosdrin and Bomyl isomers was studied. The inhibition reaction follows second order kinetics where one of the reactants (the inhibitor) is in excess. The k_2 at 25°, 30°, and 35° was determined. The energy of activation of the reaction is approximately 10 K_{cal}/mol.

The fly head enzyme is more susceptible to inhibition than the mouse brain or bovine red cell enzyme. Cis Phosdrin is about 100 times better as an inhibitor than trans Phosdrin; cis and trans Bomyl are equally good inhibitors. As expected, the rate of reactivation of any of the three enzymes is the same regardless of the dimethyl phosphate used to inhibit it. Inhibited bovine red cell, and mouse brain cholinesterases reactivate faster than the fly head enzyme.

Mouse liver homogenate degrades trans Phosdrin faster than the cis isomer, whereas cis and trans Bomyl are degraded at equal rates. Fly homogenates show no degrading activity toward any of the above compounds. Most of the mouse liver degrading activity for Phosdrin isomers was found in the supernatant fraction whereas for Bomyl isomers it was equally distributed between the microsomal and the supernatant fractions. Cis Phosdrin is degraded in a system that requires reduced glutathione, the metabolites being cis monomethyl-1-carbomethoxy-1-propen-2-yl phosphate (cis desmethyl Phosdrin) and S-methyl glutathione. On the other hand, trans Phosdrin and both Bomyl isomers were degraded to dimethyl phosphate.

Fly slices activated cis thiono Phosdrin but this property could not be found in fly homogenates or fly microsomes. Mouse liver slices, liver homogenates, or liver microsomes effectively activated cis thiono Phosdrin, in an oxygen and reduced pyridine nucleotide dependent system. In contrast, trans thiono Phosdrin was not activated to any measurable extent by fly or mouse liver preparations.

The extent of degradation of Phosdrin isomers (oxygen analogs and probable activation products of thiono Phosdrin) by mouse liver slices was found to be severely reduced by the addition to the incubation mixture of the respective thiono Phosdrin isomer.

On the basis of the present results an explanation is offered accounting for the toxicity of Phosdrin, Bomyl, thiono Phosdrin and

thiono Bomyl to the fly and the mouse.

The possible general role of glutathione in degradation of organophosphorus compounds is discussed.

INTRODUCTION

The first esterification of alcohols with phosphoric acid was probably done by Lassaigne in 1820 (1). During the following century numerous esters of phosphoric acid were synthesized but there was no mention of their poisonous nature.

In the 1930's the toxic nature of organophosphorus compounds and their potentiality as chemical warfare agents became apparent. Intensive studies were conducted in England and Germany. The work in England was directed by B. C. Saunders at Cambridge and in 1941 the well known organophosphorus compound, DFP, was synthesized. In Germany, the research was directed by Gerhard Schrader of the I. G. Farben Industries group. As early as 1934 the possible insecticidal importance of a number of these compounds was realized. Some of the organophosphorus insecticides still in use today, such as dimephox, schradan and parathion, were first synthesized by the I. G. Farben group.

After the Second World War the importance of organophosphorus compounds as insecticides became widely known, and intense research for better insecticides began. Nevertheless, their potentialities in chemical warfare continued to be studied at the Army Chemical Center,

Maryland (USA), at Porton, Wiltshire (England), at Suffield, Alberta (Canada) and also in several European countries. Fortunately, in the last 10 years there has been a liberalizing attitude in the release of the results of basic research on organophosphorus compounds, possibly because it has become evident that chemical warfare in the modern atomic world is obsolete. At the same time, more research is being carried out, particularly in industry, aimed at developing new organophosphorus pesticides of low mammalian toxicity.

It is obvious that the ability to design highly selective insecticides (highly toxic to a single species and not toxic to all other species) is proportional to our knowledge about their mode of action.

The toxic effects of many organophosphorus compounds are principally due to the inhibition of cholinesterase and the consequences of this inhibition. The anticholinesterase properties of organophosphorus compounds were first found by Adrian et al in 1941 although a report of their work did not appear until 1947 (2).

Studies on cholinesterase revealed that it has in its active center two sites, an esteratic site and an anionic site. The anionic site carries a negative charge, and binds the quaternary nitrogen of the natural substrate acetylcholine. The esteratic site is responsible for the hydrolysis of the substrate and contains several catalytically active groups, one of which is the hydroxyl group of a serine moiety. During the normal hydrolysis of acetylcholine the enzyme is acylated (acetylated) at the serine-hydroxyl group, and the choline moiety leaves the enzyme site. Finally,

the acetyl group is removed (nucleophilic attack) and the enzymatic process is completed (3-6). Organophosphorus inhibitors act in a similar way except that the serine-hydroxyl group is phosphorylated, the product being a dialkyl-phosphoryl-enzyme, where the phosphoryl group does not leave as easily as the acetyl group, with the result that the enzyme is inhibited. The inhibition is competitive because it can be prevented by the addition of acetyl choline and is also irreversible (7).

The reaction between cholinesterase and organophosphorus compounds is bimolecular and usually follows so called pseudo-first order kinetics (i.e. second order reaction where one of the reagents is in great excess). Overall, it is not merely the formation of an enzyme-inhibitor complex but represents a chemical phosphorylation resulting in the formation of a covalent bond between the enzyme and the inhibitor (7, 8).

$$(RO)_2P(O)R' + E\text{-}OH \xrightarrow{k} (RO)_2P(O)OE + R'OH$$

Mechanistically the inhibition can be considered an electrophilic attack by the organophosphorus compound on the serine-hydroxyl of the enzyme. It has been shown that the inhibitory capacity of organophosphorus compounds is correlated with the electrophilic character of the phosphorus atom (9, 10, 7). Organophosphates (P=O) are good cholinesterase inhibitors while organophosphorothionates (P=S) are weak electrophiles and very poor inhibitors of cholinesterase.

Organophosphorothionates are good <u>in vivo</u> anticholinesterase

agents due to the fact that they are oxidized or "activated" in the body to their corresponding phosphates. The activation process has been found to occur in several tissues, the liver being one of the most active. In tissue homogenates all the activity is found in the microsomal fraction and requires molecular oxygen and reduced pyridine nucleotide (7, 11015).

The activation processes of some organophosphorus compounds differ from the typical P=S to P=O oxidation. For example, schradan is activated by hydroxylation of one of its N-CH_3 groups (16), phorate and demeton by oxidation of thioether sulfur to sulfoxide and sulfone (17, 17a).

The activation process for tri-o-cresyl phosphate (TOCP) recently has been elucidated (18). It involves hydroxylation followed by cyclization with elimination of one cresyl group. One of the main characteristics of TOCP is its property of being a good in vivo inhibitor of carboxyesterase enzymes that can be responsible for the degradation (detoxication) of carboxyester-containing organophosphorus compounds. TOCP has been shown to potentiate several (19, 20).

The inhibition of cholinesterase, as mentioned before, is irreversible. Nevertheless, the enzyme can regain activity on dephosphorylation. The process is similar to the deacetylation of cholinesterase in the normal hydrolysis of acetylcholine:

$$E\text{-}O\text{-}P(O)(OR)_2 \longrightarrow E\text{-}OH + HO\text{-}P(O)(OR)_2$$

This process is known as "reactivation", and mechanistically

involves a nucleophilic attack on the phosphorylated enzyme. The reactivation rate has been shown to depend on the alkyl groups of the dialkyl-phosphoryl-enzyme. Generally a dimethyl-phosphoryl-enzyme is reactivated relatively fast, while the higher alkyl analogs are not (7, 21, 22). Nucleophilic agents, especially oximes, such as hydroxylamine, 2-PAM and bis-4-PAM have been shown to accelerate the reactivation process (23, 24, 25).

Phosphorylated cholinesterase can undergo almost complete reactivation, if this reaction proceeds immediately after the inhibition reaction, but as time passes the percent of the inhibited enzyme able to be reactivated decreases. This process is commonly referred to as "aging." The work of Berends (26) and recently of Harris et al (27) showed that during aging the inhibited enzyme loses one of the alkyl groups, and as a result a negative charge adjacent to the phosphorus atom is introduced.

$$E\text{-}O\text{-}P(O)(OR)_2 \longrightarrow E\text{-}O\text{-}\underset{\underset{O^-}{|}}{P}(O)OR$$

Consequently the inhibited enzyme is now insensitive to nucleophilic agents.

The extent to which cholinesterase is inhibited depends also on the concentration of the organophosphorus inhibitor; consequently, the toxicity of a given compound will depend on factors modifying its in vivo concentration. Storage, excretion, permeability, degradation, etc. are important factors in the toxicity of a compound. Degradation (detoxication) has been generally shown to be one of the most important.

Detoxication generally occurs as a hydrolytic process, yielding a molecule that is negatively charged (weak electrophile) and a very poor inhibitor of cholinesterase.

Most of the enzymes known to detoxify organophosphorus compounds can be classified as phosphatases (that hydrolize one or more of the phosphoric acid esters) and carboxyesterases or carboxyamidases (that hydrolize carboxyester or carboxyamide groups)(28). Also other detoxication processes have been described where no net negative charge is introduced into the organophosphorus molecule, such as the reductive detoxication of parathion (29) and the oxidative degradation of amiton (30).

The scheme presented in Figure 1 summarizes the main factors affecting the toxicity of organophosphorus compounds. It must be pointed out that the importance of any one of these factors in determining toxicity could vary between different compounds and also between different animal species. The study of these factors and their relation to toxicity could lead to the development of highly specific insecticides and also to a better understanding of the toxicity of known organophosphorus compounds.

The first organophosphorus compounds used as insecticides were equally toxic to insects and mammals (e.g. parathion). The introduction of malathion (O, O-dimethyl-S-1, 2-dicarbethoxyethyl phosphorodithioate) initiated a series of insecticides of low mammalian toxicity. This

selective toxicity was attributed to a more extensive degradation of these compounds by the mammal, involving the hydrolysis of a carboxyester group (31). O'Brien (32, 33) showed that selectivity was more or less a general property of carboxyester-containing organophosphorus compounds, the thiono analog being more selective than the corresponding oxygen analog. This difference was explained by the fact that in the phosphorothioate there is a lag period required for the production of the cholinesterase inhibitor, the phosphate, by P=S oxidation. This lag period gives an opportunity for detoxifying mechanisms to operate.

Although most carboxyester-containing organophosphorus compounds show some selectivity, the reverse is not necessarily true. Examples of compounds that do not contain carboxyester groups but are nevertheless selective include Sumithion (34, 35, 59a, 59b, 59c), Diazinon and Dipterex (35a). Undoubtedly, selectivity could arise because of differences in any of the factors shown in Figure I. Recently, resistance (which can be regarded as a special case of selectivity) to organophosphates in the "two spotted mite" has been shown to be caused by natural selection of a cholinesterase resistant to inhibition by organophosphorus compounds (36).

The cis and trans geometrical isomers of a carboxyester-containing vinyl phosphate, mevinphos, dimethyl 1-carbomethoxy-1-propen-2-yl phosphate (Phosdrin) and its thiono analog, dimethyl-1-carbomethoxy-1-propen-2-yl phosphorothionate (thiono Phosdrin) show slight selectivity (37). The toxicological data of the Phosdrin isomers also offer an

interesting example of how different the biological properties of a compound can be, as a consequence of a different spatial arrangement of certain groups. Cis Phosdrin is about 50 times more toxic to the fly and 20 times more toxic to the mouse than trans Phosdrin (37).

A new vinyl organophosphorus insecticide, dimethyl 1, 3-dicarbomethoxy-1-propen-2-yl phosphate (Bomyl), is structurally related to Phosdrin. Because of this structural similarity it was considered of interest to separate the geometrical isomers of Bomyl, synthesize the thiono Bomyl isomers and compare their toxicities with those of the Phosdrin and thiono Phosdrin isomers. Also, it was considered important to study the inhibition of insect and mammalian cholinesterases by Phosdrin and Bomyl isomers, as well as the degradation of these compounds and the activation of their respective sulfur analog in order to gain a better understanding of the basis of their toxic properties.

The chemical structures of Phosdrin, Bomyl, thiono Phosdrin and thiono Bomyl isomers are presented in Figure 2.

FIGURE 1

Scheme of main factors affecting the toxicity of organophosphage and organophosphorothionate compounds.

When cholinesterase is inhibited beyond a critical level, intoxication symptoms and death occur.

E-OH	Active cholinesterase.
$O=P(OR)_3$	Organophosphate (very good inhibitor and electrophilic agent).
$S=P(OR)_3$	Organophosphorothionate (very poor inhibitor and electrophilic agent).
$E-O-P(O)(OR)_2$	Inhibited cholinesterase.
E-O-P(O) OR O-	Aged inhibited-cholinesterase (not susceptible to nucleophilic attack).

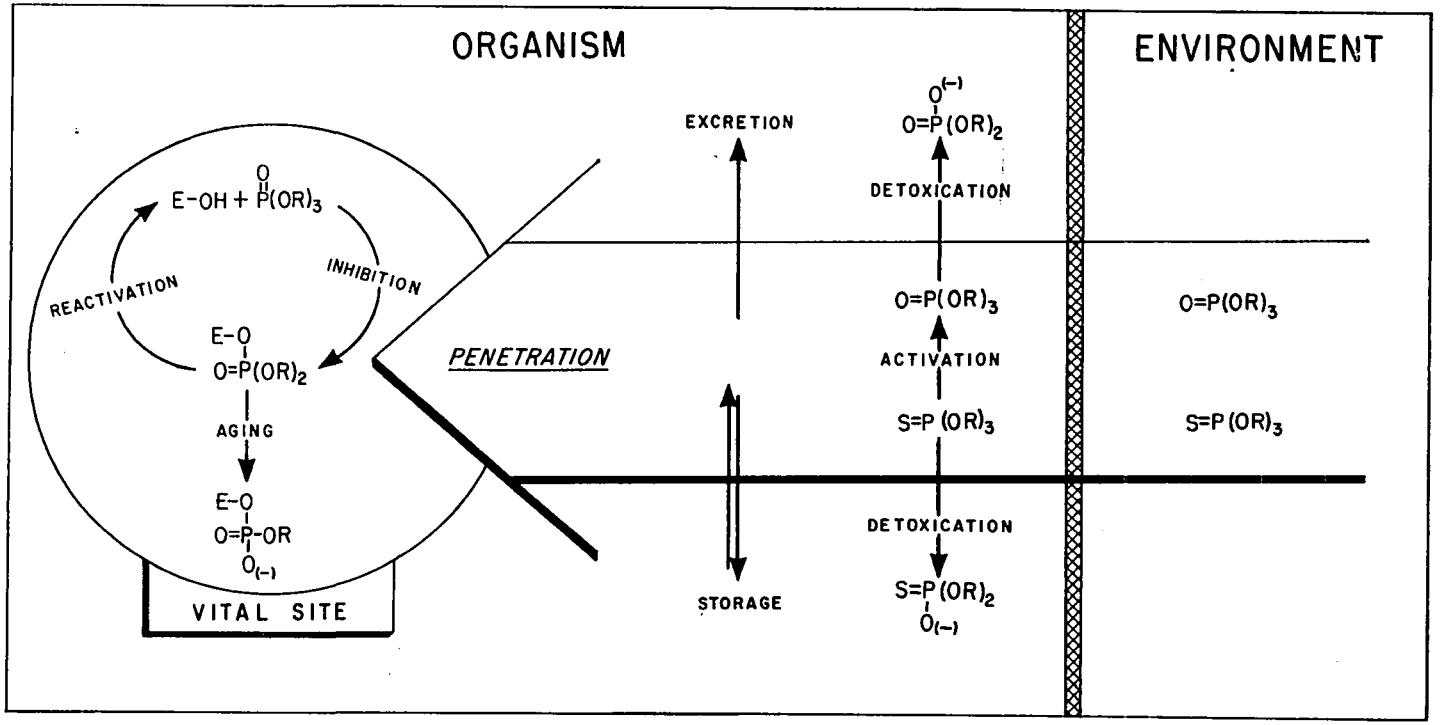
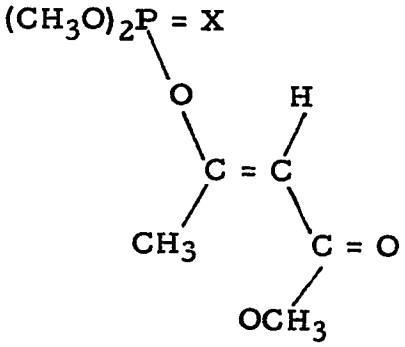

ORGANISM
ENVIRONMENT
E-OH + P(OR)3
INHIBITION
REACTIVATION
E-O
O=P(OR)2
AGING
E-O
O=P-OR
O(-)
VITAL SITE
PENETRATION
EXCRETION
STORAGE
O(-)
O=P(OR)2
DETOXICATION
O=P(OR)3
ACTIVATION
S=P(OR)3
DETOXICATION
S=P(OR)2
O(-)
O=P(OR)3
S=P(OR)3

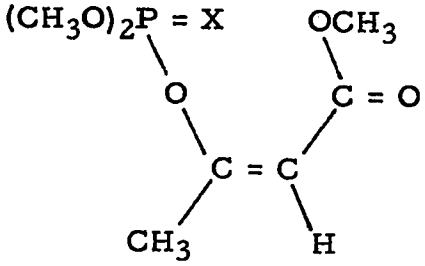
FIGURE 1

Figure 2: Structures of Phosdrin, Bomyl, thiono Phosdrin and thiono Bomyl isomers.



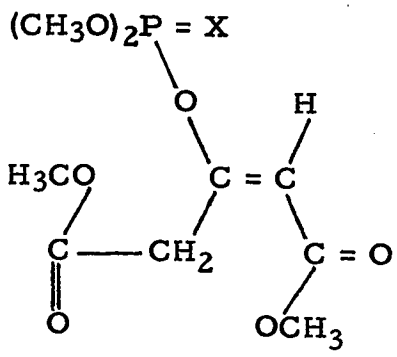
cis Phosdrin (X = O)

cis thiono Phosdrin (X = S)



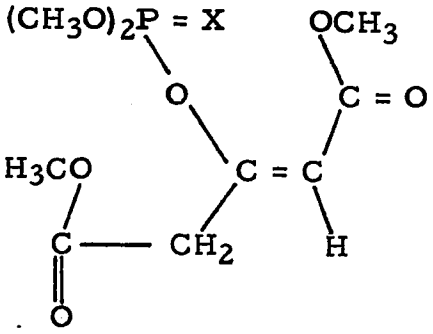
trans Phosdrin (X = O)

trans thiono Phosdrin (X = S)



cis Bomyl (X = O)

cis thiono Bomyl (X = S)



trans Bomyl (X = O)

trans thiono Bomyl (X = S)

MATERIALS

1. ANIMALS - The mice were female albinos, supplied by Rolfsmeyer Frams, Madison, Wis. and kept in our laboratory for at least a week (20-25 g live weight) before use, except when otherwise indicated.

Three day old susceptible houseflies, Musca domestica L., bred in this laboratory were used throughout.

2. INSTRUMENTS - Craig counter-current. Glass unit made by H.O. Post Scientific Instrument Co., 6822-60th Road, Maspeth 78, N.Y.

Ultracentrifuge. Spinco Model L.

Infra-red. Recording Infrared Spectrophotometer, Model 21, Perkin-Elmer Corporation. Sodium chloride cells (0.1 mm) were used.

3. CHEMICALS - Technical samples of Phosdrin and Bomyl provided by Shell Chemical Co. and Allied Chemical Co., respectively, were the starting materials used to obtain the different geometrical organophosphate isomers.

TOCP and glutathione were products of Eastman Organic Chemical, methylglutathione was supplied by Calbiochem. Bovine red cell cholinesterase was a product of Sigma Chemical Co. All other chemicals used were of the best quality commercially available.

METHODS

1. PREPARATION OF ORGANOPHOSPHORUS COMPOUNDS

a) Phosdrin and Bomyl Isomers. Craig counter-current distribution was used to separate the isomers. A redistilled sample (5g) of technical Phosdrin or Bomyl was used in the ether-water system (10ml phases) described by Spencer et al (38). After 60 transfers, the water phase of every fourth tube was sampled and analyzed for phosphorus (39) to determine the location of each isomer.

Cis Phosdrin was obtained from tubes 21-36, which were combined and evaporated under a vacuum, and the trans isomer was obtained from tubes 47-59. The yield of each isomer and the respective boiling points were approximately the same as those reported by Spencer et al (38).

For Bomyl, tubes 11-27 yielded the cis isomer (2.7g; b.p. 130-131°/0.05 Torr.) and tubes 34-54 yielded the trans isomer (1.4g; b.p. 130-131°/0.05 Torr.). The cis and trans configurations were assigned to Bomyl isomers by comparison of their polarity and infrared spectra with those of Phosdrin. For Phosdrin isomers the configuration has been established from an examination of magnetic resonance spectra (40, 60). The rules proposed by Schmerling (41) are used here to designate the specific cis and trans isomers.

Infrared spectra were determined at 10% (w/v) concentration in CCl_4 (Figures 3 and 4). The absorption bands found useful to characterize and differentiate the isomers were:

For Phosdrin:	cis isomer	1655	1245	1140	950	895	cm^{-1}	
	trans isomer	1680	1220	1160	980	915	cm^{-1}	
For Bomyl:	cis isomer	1655	1370	1325	980	945	895	cm^{-1}
	trans isomer	1670	1345			950		cm^{-1}

The isomers were stored in a deep freeze and periodic checks were made using infrared analysis. By contrast with Phosdrin, Bomyl readily isomerizes in light at room temperature.

b) Thiono Phosdrin Isomers. The preparation is basically the same as that reported by Stothers and Spencer (40).

Trans thiono Phosdrin

Redistilled methylacetoacetate (0.1 moles) was dissolved in 160ml of benzene, and metallic sodium (0.1 moles) added at room temperature with stirring under anhydrous conditions. The mixture was refluxed overnight (18 hrs). The preparation was cooled to around 45° and redistilled dimethylphosphorochloridothionate (0.1 moles) was added during a 30 minute period. The mixture was refluxed overnight (20 hrs), cooled to room temperature and washed twice with 100ml of water. The water layers were discarded and the benzene solution dried over anhydrous sodium sulfate. The benzene was evaporated from the orange solution and the resulting oil fractionated. The fraction distilling

between 85°-95° (0.4 Torr.) crystallized on cooling. The crystals were washed twice with cool hexane and dried at room temperature under vacuum. The colorless crystals were trans thiono Phosdrin (MP=46°-48°) as indicated by infrared spectrum analysis. The overall yield of trans thiono Phosdrin was approximately 15%.

Cis Thiono Phosdrin

One gram of trans thiono Phosdrin dissolved in 10ml of carbon tetrachloride was irradiated with ultraviolet light (Hanovia Chemical and M.G.G. Co., 435 Watts). Isomerization to an equilibrium mixture occurred in 15 hrs as shown by infrared analysis. The carbon tetrachloride was evaporated under vacuum and the residue fractionated on an alumina column (50g of alumina grade I + 2% water; 1cm diameter). The column was eluted successively with 75ml of hexane, 125ml of carbon tetrachloride and 100ml of chloroform. Sixty, five mililiter fractions were collected and every fourth tube was sampled and analyzed for phosphorus. Tubes 30 to 46 were combined and on evaporation of solvent a slightly yellow oil was obtained. This oil was vacuum distilled (0.1 Torr.) yielding a colorless liquid with infrared spectrum corresponding to cis thiono Phosdrin. The overall yield was approximately 40%. Figure 5 shows the infrared spectra of cis and trans thiono Phosdrin (10% w/v in CCl_4).

c) Cis Desmethyl Phosdrin. Cis desmethyl Phosdrin was synthesized by reacting cis Phosdrin with sodium iodide as described by Spencer et al (38).

d) Cis Thiono Bomyl. Redistilled dimethyl-acetone-dicarboxylate (0.1 moles) was dissolved in 200ml of ether, and metallic sodium (0.1 moles) was added at room temperature during a one hour period, with stirring under anhydrous conditions. After the sodium addition was completed, redistilled dimethylphosphorochloridothionate (0.1 moles) was added and the mixture refluxed for six hours. The preparation was cooled to room temperature and the ether evaporated under vacuum. The remaining oil was dissolved in 150ml of chloroform and extracted three times with 150ml of water. The water layers were discarded and the chloroform dried over anhydrous sodium sulfate. The chloroform was evaporated from the reddish solution under vacuum and the resulting oil fractionated using the Craig counter-current technique with an ether/water system, 60 transfers, as previously described. Several factions were isolated and their infrared spectra determined. The fraction between tubes 48 to 60 was found to have a spectrum similar to cis Bomyl except for P=S absorption replacing P=O. Part of this fraction (1.5g) was further fractionated in an alumina column (100g of alumina grade 1 + 1% water; 2.8cm diameter). The column was eluted successively with 100ml of hexane, 250ml of chloroform. Sixty 10 mililiter fractions were collected. Tubes 44 to 49 were combined and on evaporation of solvent yielded a slightly yellow oil thought to be cis thiono Bomyl because of the following considerations:

(a) It showed an infrared spectrum very similar to cis Bomyl except for the absence of the strong p=O band (1275-1300cm^{-1}).

(b) Under mild oxidation conditions (CrO_3 in water) it was changed to a compound of identical infrared spectrum to cis Bomyl (yield = 85-90%). The overall yield of cis thiono Bomyl was approximately 2%. In contrast to thiono Phosdrin, attempts to obtain trans thiono Bomyl by isomerization of the cis isomer using ultraviolet light or triethylamine were unsuccessful. Figure 6 shows the infrared spectrum of cis thiono Bomyl (10% w/v in CCl_4).

Another attempt to synthesize thiono Bomyl was as follows: β-chloro glutaconic acid was prepared by reacting dimethyl acetone dicarboxylate with phosphorus pentachloride. Both carboxylic groups of β-chloro glutaconic acid were esterified with methanol. Theoretically, the dimethyl ester of β-chloro glutaconic acid on reacting with sodium dimethyl phosphorothionate should eliminate sodium chloride and produce thiono Bomyl. The latter reaction did not take place, as it was not possible to isolate thiono Bomyl from the reaction mixture.

The major difficulty encountered in the synthesis and purification of cis thiono Bomyl was probably due to its physical characteristics. It is an oil that decomposes on distillation.

2. TOXICITY DETERMINATIONS

Toxicity of the compounds to the mouse was determined by intraperitoneal injection of 0.2ml of water for the phosphates and in 0.2ml of corn oil for the thiono phosphates and TOCP.

TOCP was injected four hours previous to the injection of the other organophosphorus compounds to allow for activation. Mortality was recorded after 24 hours although no change occurred after 1 hour for Phosdrin, Bomyl and malaoxon or after 10 hours for thiono Phosdrin and thiono Bomyl. Control groups injected with water, corn oil or TOCP showed no mortality under the above experimental conditions. Groups of 20 mice were used.

Toxicity against female flies was determined by topical application of 1µl of an acetone solution to the thorax. Mortality was recorded after 24 hr. Groups of 20 flies were used.

3. INHIBITION AND REACTIVATION OF CHOLINESTERASES

a) Sources of Cholinesterases. A homogenate containing 80mg of mouse brain or 6 fly heads per ml of phosphate buffer (0.001M, pH 7.5) freshly prepared was centrifuged at 1700g for 3 min. to remove the debris. The supernatant was used as a source of cholinesterase. For the bovine red cell enzyme a water solution of a commercial preparation (see Materials) was used.

b) Determination of Cholinesterase Activity. Cholinesterase activity was determined using a manometric technique. Warburg flasks contained 0.01M phosphate buffer (pH 7.5), 0.01M, $MgCl_2$; 0.03M, $NaHCO_3$ as well as cholinesterase and inhibitor, in a total volume of 2.8ml. The vessels were gassed with a mixture of 95% nitrogen: 5% carbon dioxide for 10 minutes. After a specified period of

preincubation (inhibition time) acetyl choline bromide (10mg in 0.2ml) was tipped from the side arm and readings were taken at regular intervals throughout the following 40 minutes. Percent acetyl cholinesterase activity was calculated on the basis of this assay. Under these experimental conditions flasks containing uninhibited enzyme evolved 110-130µl of CO_2 in 30 min at 25°.

This assay was used to study the inhibition of cholinesterases by the organophosphorus compounds, and also to evaluate the quantity of organophosphates degraded or produced in the degradation and activation studies.

c) Reactivation of Cholinesterases. For the reactivation studies, the same manometric technique as above was used, but the temperature was 35°. The evolution of CO_2 was recorded every 10 min. for 70 or 90 min. The percent inhibition was calculated for each 10 min. interval.

4. DEGRADATION OF PHOSDRIN AND BOMYL ISOMERS

a) Degradation of Phosdrin and Bomyl Isomers by Mouse Liver and Fly Fractions. Incubations were carried out in 10ml Erlenmeyer flasks in a water-bath shaker. Mouse liver homogenates were prepared in 0.25M sucrose using a Potter-Elvehjem unit fitted with a Teflon pestle. Fly homogenates were prepared in 0.25M sucrose, 0.01M phosphate buffer (pH 7.5) by grinding decapitated flies (mixed sexes) in a mortar. The resulting homogenate was filtered through cheesecloth

before use. Part of the homogenate was centrifuged at 10,000 x g for 10 min. The pellet was discarded and the supernatant centrifuged at 100,000 x g for 30 min. The microsomal pellet and the supernatant were used in the degradation studies. In the experiments on the effects of glutathione and aging, mouse liver homogenates were prepared in 0.25M sucrose or 0.9% sodium chloride (1:4, w:v) and centrifuged at 10,000 x g for 10 min. The supernatant was divided into three portions, one of which was assayed immediately for Phosdrin and Bomyl degradation. The second portion (5ml) was dialized for 6 hours in the cold against 0.25M sucrose, 0.001M phosphate buffer (pH 7.5) or 0.9% sodium chloride 0.001M phosphate buffer (pH 7.5) with one change of the dializing medium after 3 hours. The third portion was kept as such and assayed together with the dialized liver supernatant for Phosdrin and Bomyl degradation.

After incubation, appropriate dilutions were made and an aliquot was tested against bovine red cell cholinesterase at 25° to evaluate the quantity of organophosphate remaining.

b) Procedure for identification of the glutathione metabolite. Mouse liver homogenates were prepared in sodium chloride (1:4; w:v) and centrifuged at 45,000 x g for 1 hour. The supernatant (35ml) was dialyzed, as described above, against 0.9% NaCl containing 0.1% mercaptoethanol.

Each incubation flask contained dialized mouse liver supernatant

equivalent to 7g wet weight; cis Phosdrin, 1.4mM; reduced glutathione, 3.2mM; tris-buffer, 20mM (pH 7.5). The total volume was 50ml. The mixture was incubated at 35° for 40 min. At the end of incubation, the mixture was boiled for 3 min., filtered, concentrated to 3ml under vacuum at 30° and filtered again. The filtrate (referred to below as "product of the reaction") was used for chromatography. Treatment with iodoacetamide was done as reported by Johnson (42).

(c) Chromatographic Identification of Organophosphate Metabolites.

Phosdrin and Bomyl Isomers-Acid Procedure

Mouse liver homogenates were prepared in 0.9% sodium chloride (1:4; w:v) and centrifuged at 10,000 x g for 10 min. The supernatant was used throughout.

Each incubation flask contained mouse liver supernatant equivalent to 3g wet weight; tris-buffer, 0.5 millimoles (pH 7.5) and organophosphate, 20mg. When cis Phosdrin was the organophosphate, 100mg of glutathione was added to the incubation system. The mixture was incubated at 35° for one hour in a total volume of 10 ml.

The reaction was stopped by adding 0.5ml of 6N HCl and 100ml of acetone. The mixture was filtered through Whatman No. 42 filter paper. The filtrate was concentrated to 5ml under vacuum at 30° and filtered again. The pH of the solution was adjusted to less than one with 6N HCl. The solution was then extracted twice with 20 ml of chloroform.

The chloroform layer was concentrated under vacuum and used for chromatography. The water layer was also concentrated under vacuum, applied on Whatman No. 1 paper strips (2cm wide) and eluted (descending) with 95% acetone. The eluate (about 1ml) was used for chromatography. Chromatographic solvents are given in Tables 11 and 12. The chromatographic spots were located by spraying with acid and ammonium molybdate (43), drying at room temperature and exposing to ultraviolet light for 2 min.

Cis Phosdrin-Neutral Procedure

The incubation was done as above, with the exception that sodium phosphate buffer was used instead of tris-buffer. The reaction was stopped by adding 50ml of acetone. The mixture was filtered, concentrated under vacuum to approximately 5ml and centrifuged at 45,000 x g for 2 hours. The supernatant was further concentrated under vacuum, applied on paper strips, and eluted with 80% acetone as above. The eluate was used for chromatography.

5. ACTIVATION OF THIONO PHOSDRIN ISOMERS

a) Mouse Liver and Fly Slices. Mice were killed by decapitation and livers were removed and washed with cold 0.9% KCl. Liver slices were prepared with the aid of a Stadie-Riggs microtome.

Flies were chilled, decapitated, and longitudinally cut in half with the aid of a sharp blade.

b) Mouse Liver and Fly Homogenates, Supernates and Microsomes. Mouse liver homogenates were prepared in 0.25M sucrose using a Potter-Elvehjem unit fitted with a Teflon pestle. Fly homogenates were prepared in 0.25M sucrose, 0.0125M phosphate buffer (pH 7.5) by grinding decapitated flies (mixed sexes) in a mortar. The resulting homogenates were filtered through cheesecloth before use.

Mouse liver or fly homogenates prepared as described above were centrifuged at 10,000 x g for 10 min. The resulting supernatants were spun at 100,000 x g for 30 min. The pellets (microsomes) were resuspended in 0.25M sucrose, using a Potter Elvehjem unit.

c) Incubation Procedure. Water solutions of organophosphorus compounds were prepared fresh each day. Water suspensions of thiono Phosdrin isomers and cis thiono Bomyl were prepared with the aid of an equal amount of Atlas detergent 8-916P.

Most incubations were carried out in 10ml-Erlenmeyer flasks open to the air, in a water bath shaker. In those experiments where the influence of oxygen on microsomal metabolism was studied, a Warburg water bath was used. The Warburg flasks contained all components of the incubation system in the main vessel except for the microsomal suspension that was put in the side arm. After gassing with nitrogen for 20 min. the microsomal fraction was tipped from the side arm and the incubation carried on. Gassing was continued through the incubation period. Two kinds of nitrogen were used: a) commercial

nitrogen, direct from the cylinder, and b) commercial nitrogen passed through two traps containing Fieser's solution (44). After incubation, the mixtures were put in a boiling water bath for 3 min. An aliquot was tested against bovine red cell cholinesterase at 25°. The amount of organophosphates present in the incubation mixtures was calculated from standard curves of cholinesterase inhibition (inhibitor concentration versus perdent inhibition).

6. EXPERIMENTAL VARIATION

All results presented in this thesis are averages of three or more experiments. Variation between experiments was not more than five percent.

FIGURE 3

Infra-red spectra of Phosdrin isomers

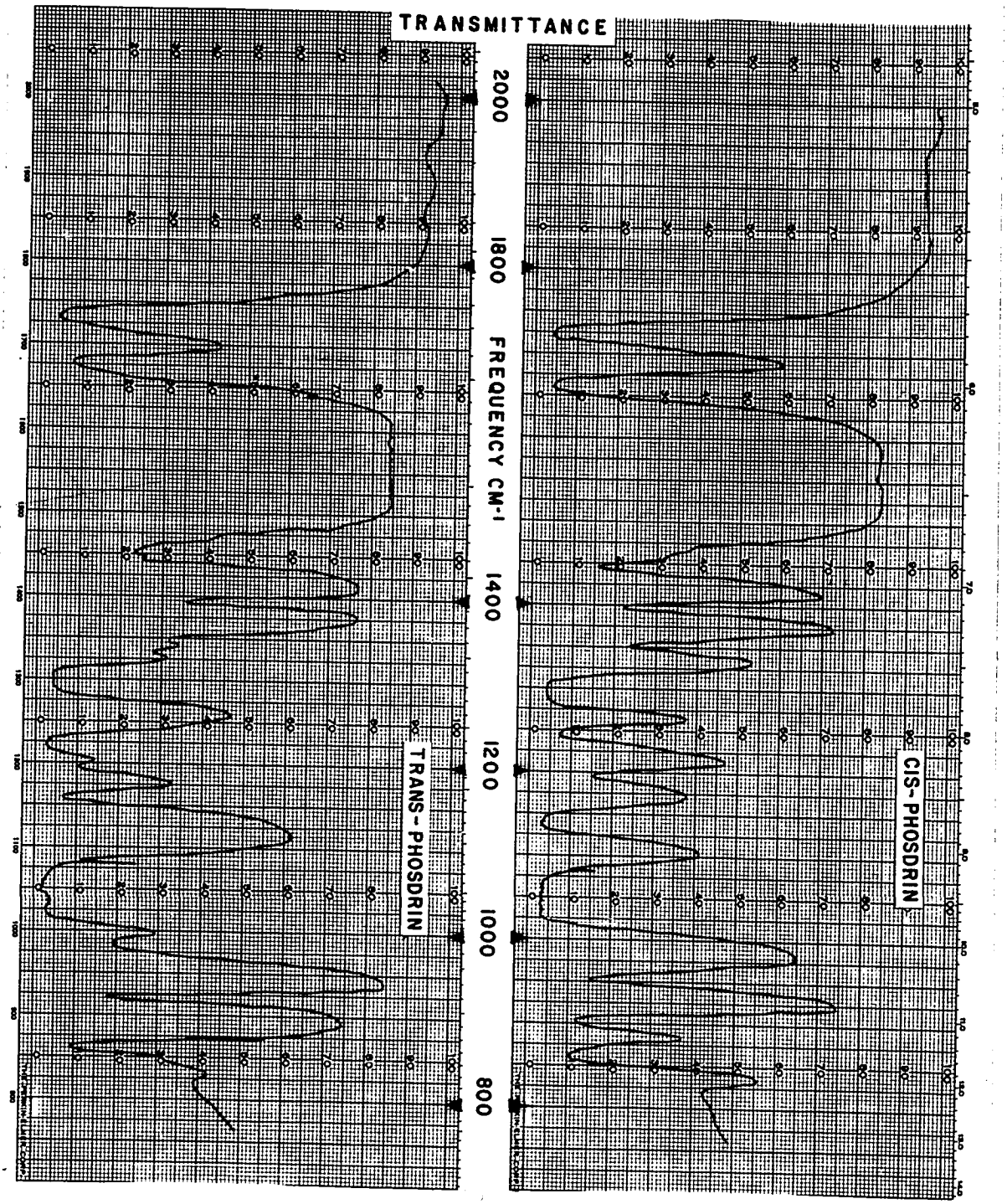


FIGURE 3

Infra-red spectra of Phosdrin isomers

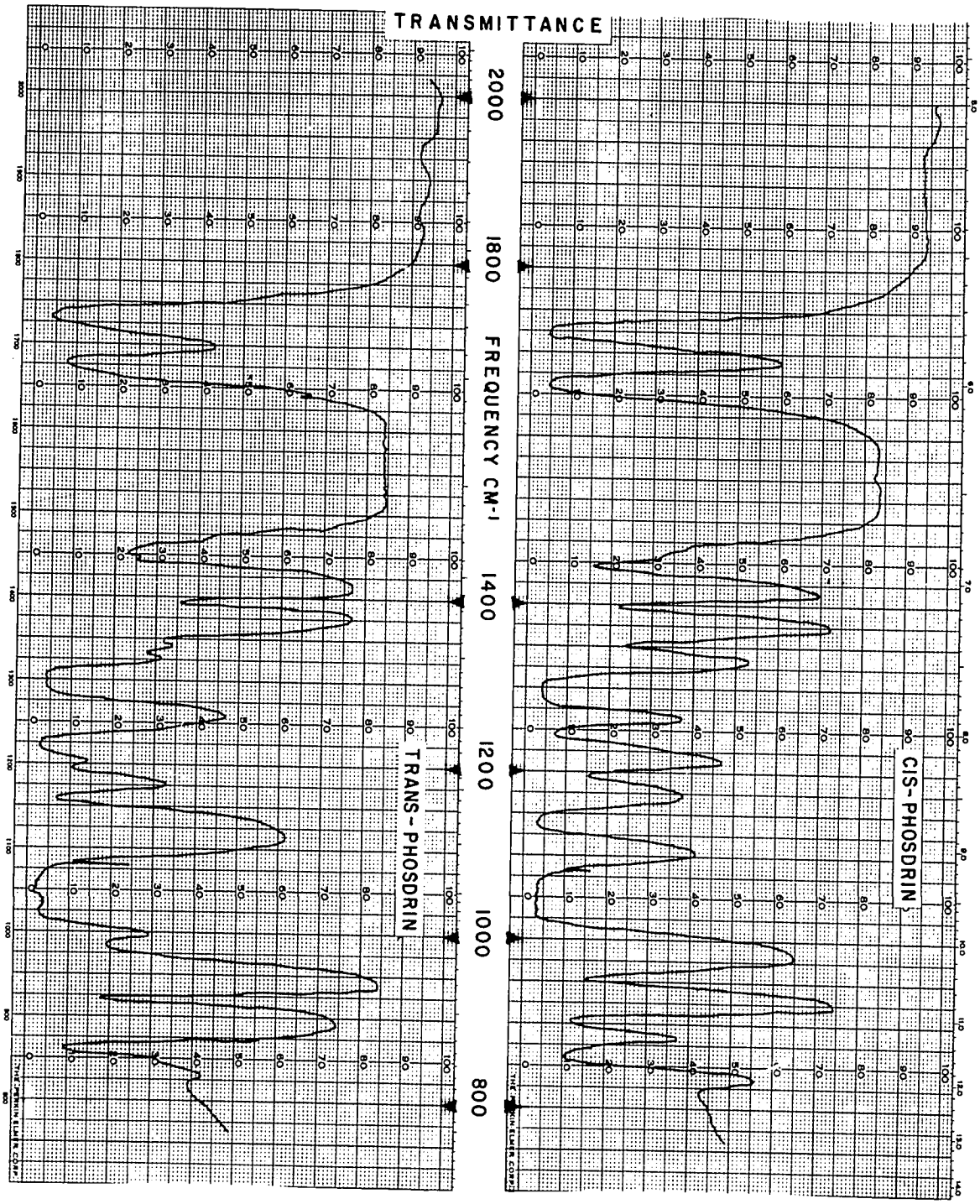


FIGURE 4

Infra-red spectra of Bomyl isomers

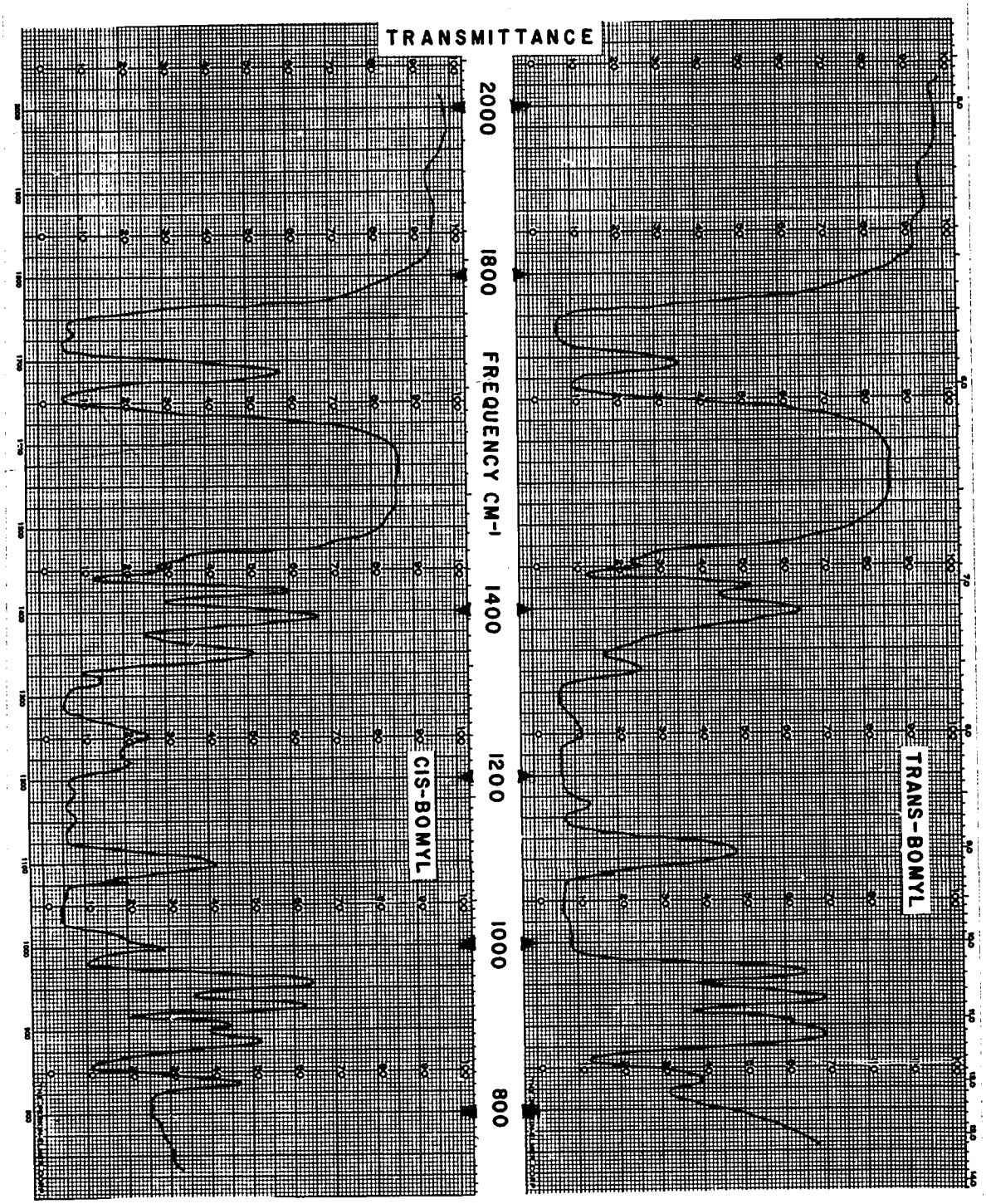


FIGURE 4

Infra-red spectra of Bomyl isomers

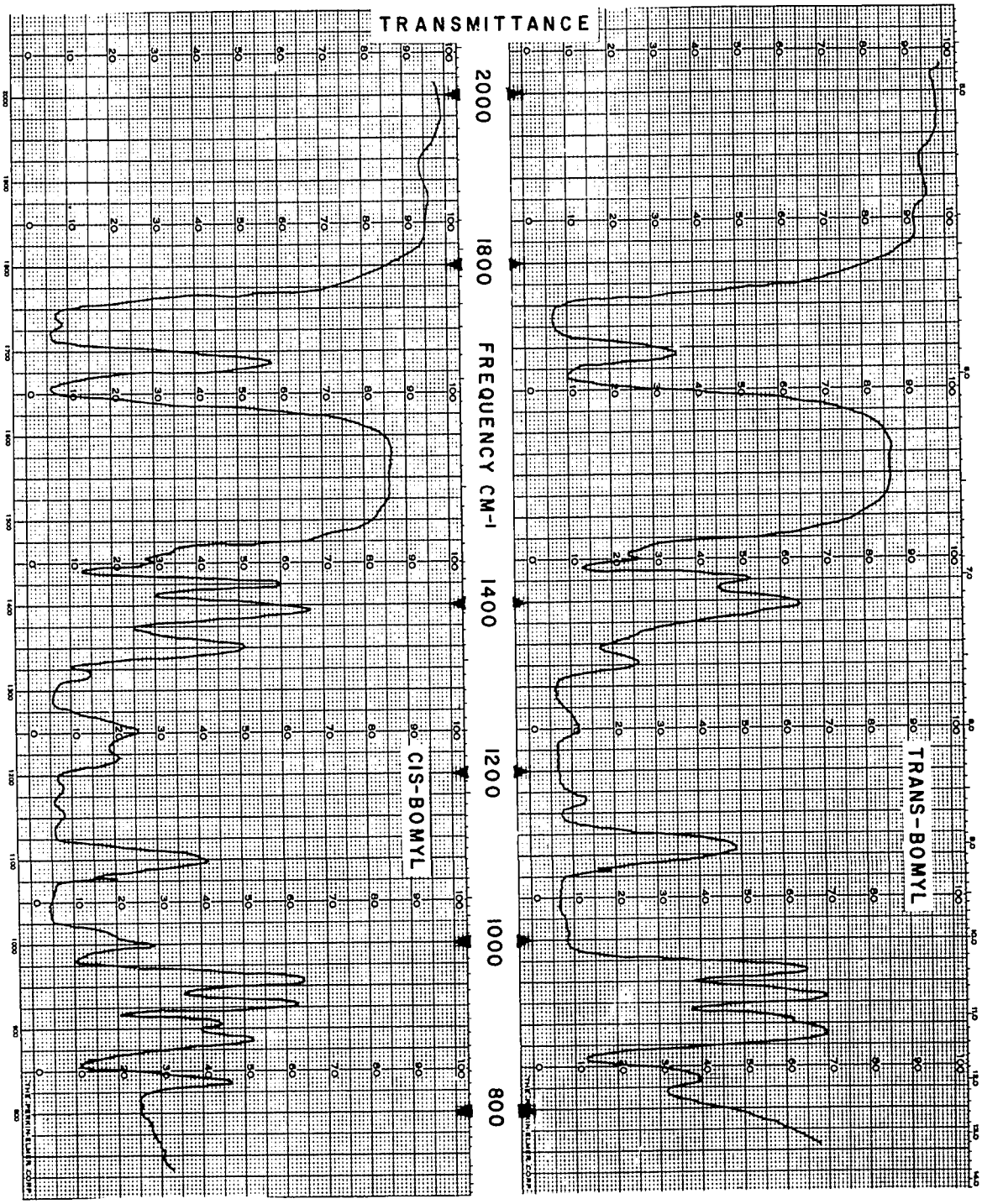


FIGURE 5

Infra-red spectra of thiono Phosdrin isomers.

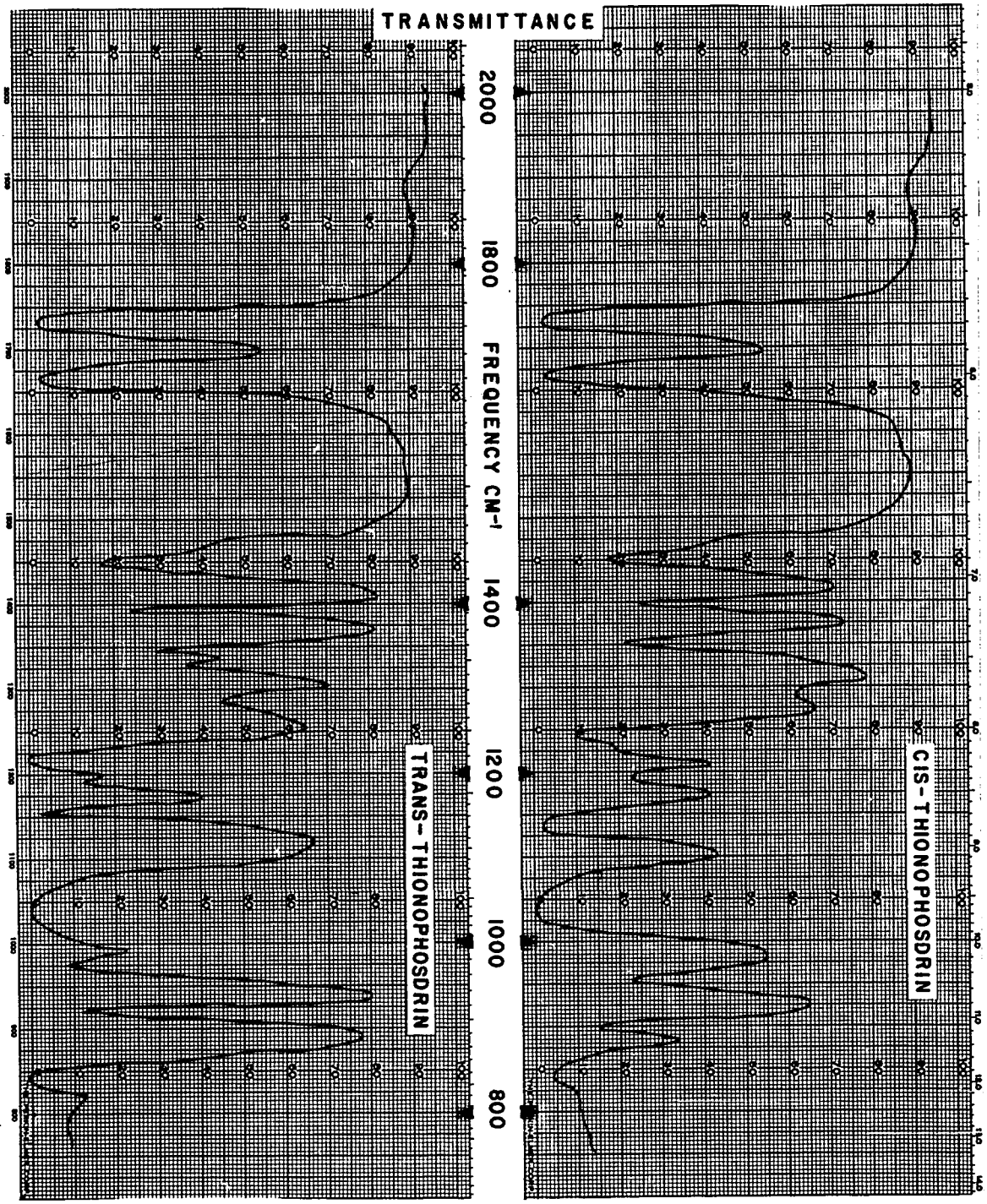


FIGURE 5

Infra-red spectra of thiono Phosdrin isomers.

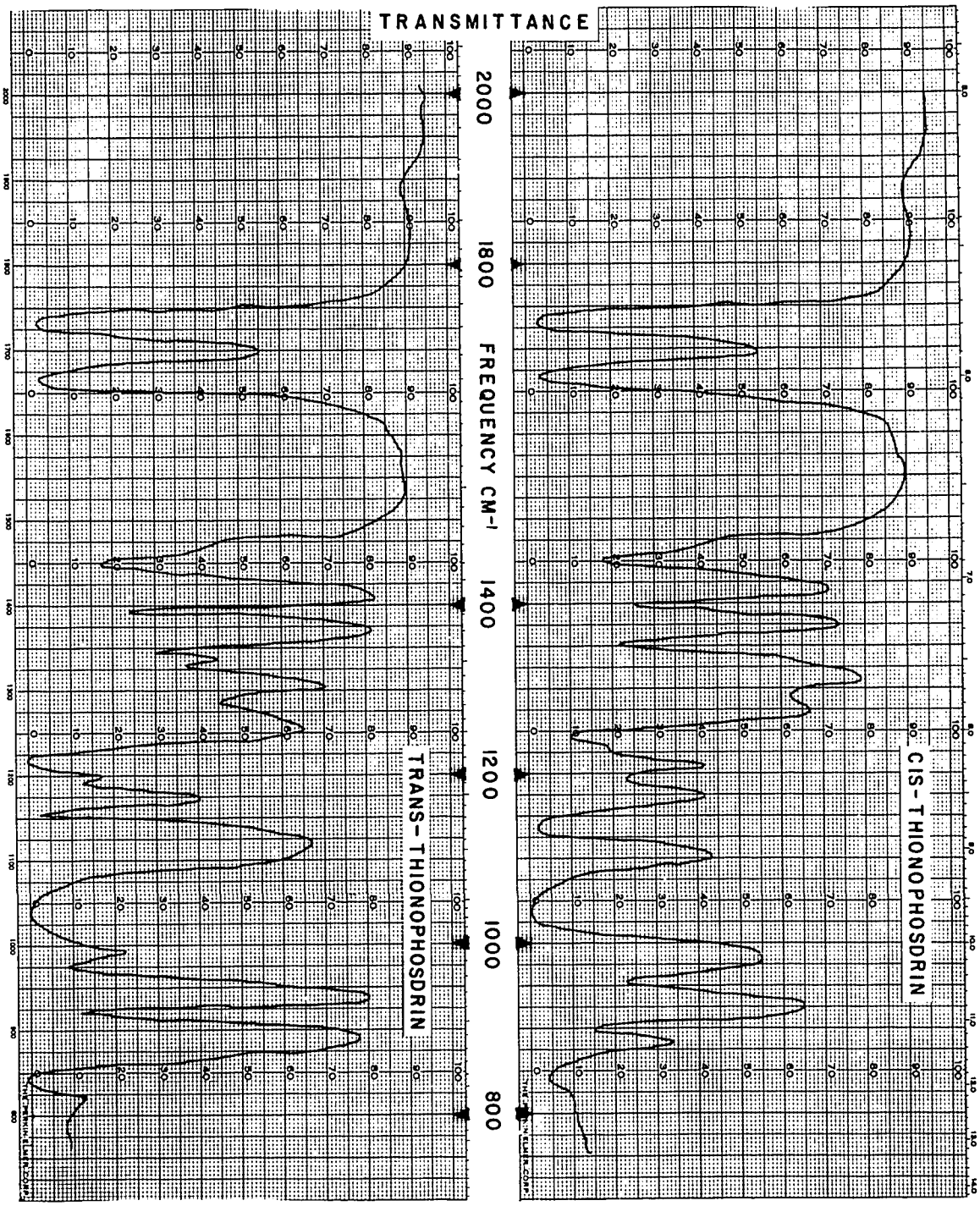


FIGURE 6

Infra-red spectrum of cis thiono Bomyl.

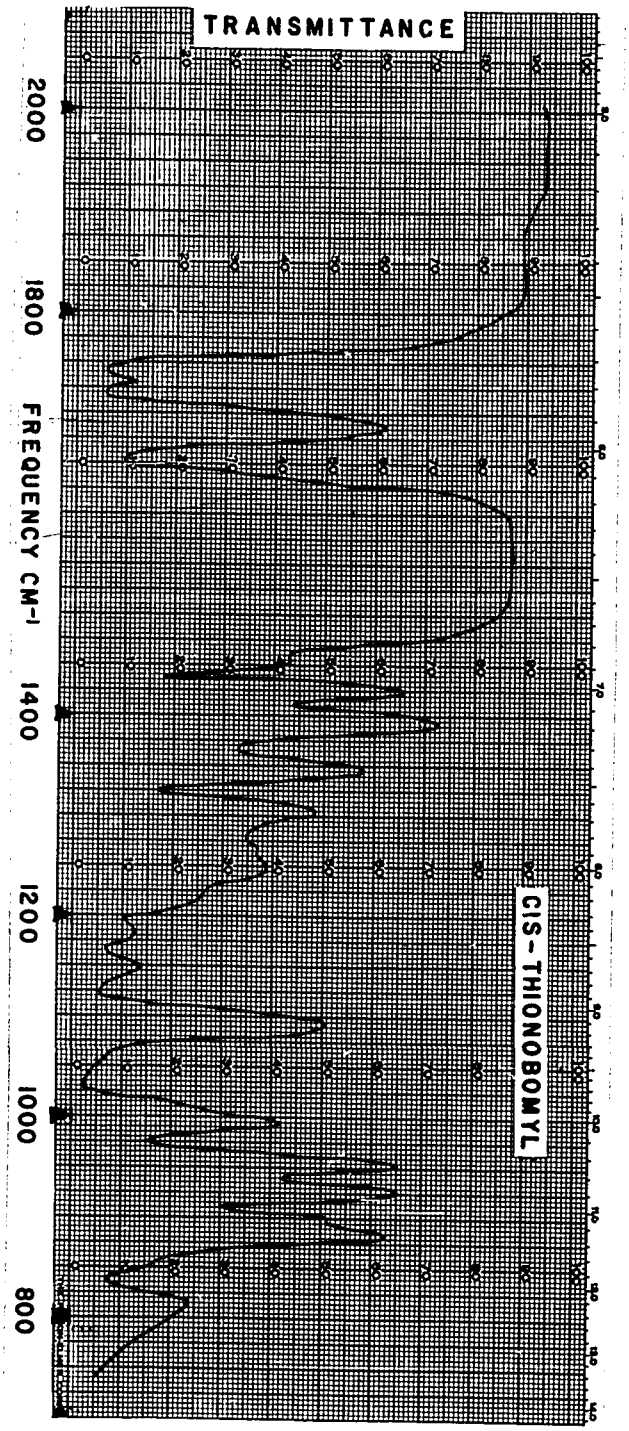
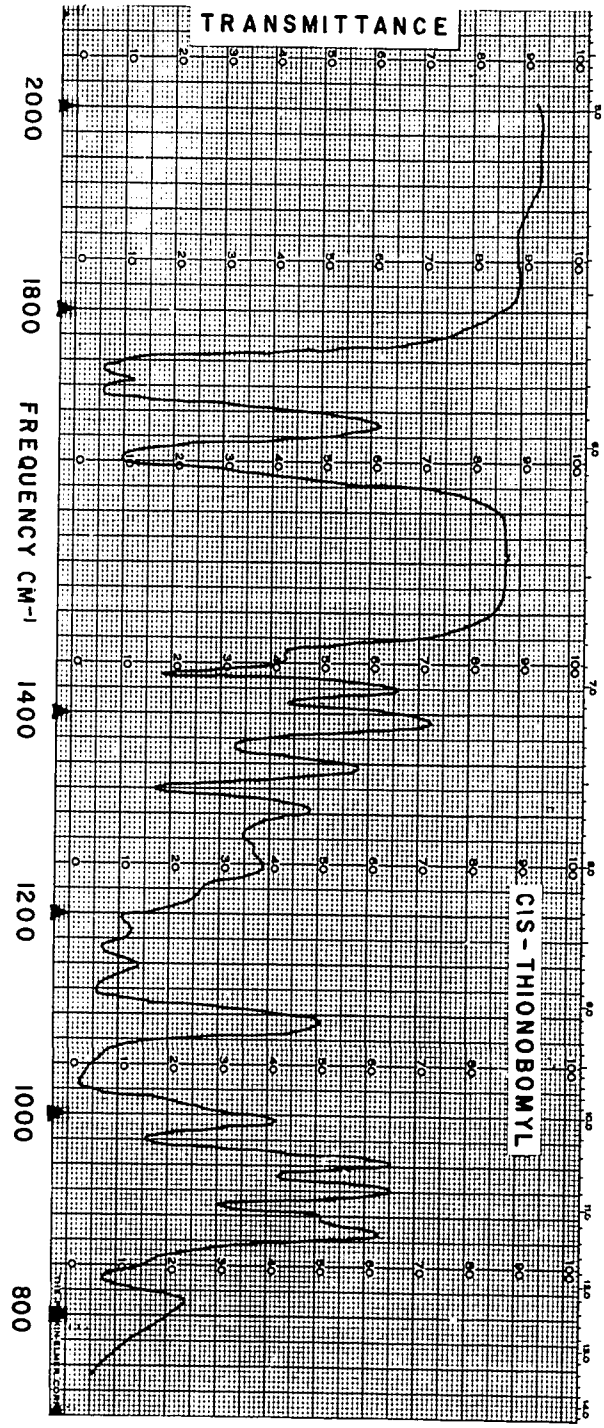


FIGURE 6

Infra-red spectrum of cis thiono Bomyl.



RESULTS

1. TOXICITY

Table 1 shows the toxicity of Phosdrin and Bomyl isomers to the fly and the mouse. Cis Phosdrin is more toxic than the trans isomer to both species. By contrast, trans Bomyl is as toxic as the cis isomer. Trans Phosdrin and both Bomyl isomers show no selectivity, being equally toxic to the fly and the mouse. On the other hand, cis Phosdrin is approximately seven times more toxic to the fly than to the mouse.

Table 2 shows the toxicity of thiono Phosdrin isomers and cis thiono Bomyl to the fly and the mouse. All three compounds are more toxic to the fly than to the mouse. Cis thiono Phosdrin is more toxic than the trans isomer to both species.

It is interesting to note that in contrast with Phosdrin and Bomyl isomers, thiono Phosdrin and cis thiono Bomyl are more selective, as judged for the toxicity ratios (mouse/fly).

The toxicological data for Phosdrin and thiono Phosdrin isomers presented in Tables 1 and 2 are in good agreement with that reported by Spencer (37) except for the higher LD_{50} for trans Phosdrin and thiono Phosdrin isomers to the fly.

TABLE 1

TOXICITY OF PHOSDRIN AND BOMYL ISOMERS[1]

	LD_{50} (mg/kg)		Toxicity Ratio Mouse/Fly
	Fly	Mouse	
Cis Phosdrin	0.27	2.0	7.4
Trans Phosdrin	45.0	45.0	1.0
Cis Bomyl	2.3	1.6	0.7
Trans Bomyl	2.4	1.4	0.6

[1] Topical application to the fly and intraperitoneal injection into the mouse (see Methods).

TABLE 2

TOXICITY OF THIONO PHOSDRIN ISOMERS AND CIS THIONO BOMYL

	LD_{50} (mg/Kg)[1]		Toxicity Ratio Mouse/Fly
	Fly	Mouse	
Cis thiono Phosdrin	3.2	43	13
Trans thiono Phosdrin	22.0	930	42
Cis thiono Bomyl	2.8	56	20

[1] Topical application to the fly and intraperitoneal injection into the mouse (see Methods).

2. INHIBITION AND REACTIVATION OF CHOLINESTERASES

a) Inhibition of cholinesterases by Phosdrin and Bomyl Isomers.

Figure 7 shows the kinetics of the inhibition of bovine red cell and fly-head cholinesterases with cis and trans Phosdrin. The log percent activity is inversely proportional to the inhibitor concentration at a constant time and also proportional to the inhibition time at a constant inhibitor concentration (solid line). When the concentration of the enzyme was varied three fold the percent inhibition was constant, for a constant inhibitor concentration and inhibition time.

The above results are in agreement with the postulate that the inhibition reaction is bimolecular where one of the reactants is in great excess:

$$E + I \xrightarrow{k_2} EI$$

$$k_2 = \frac{2.303}{t([I] - [E])} \log \frac{[E]([I] - [x])}{[I]([E] - [x])}$$

$$[I] \gg [E]$$

$$\therefore \quad k_2 = \frac{2.303}{t[I]} \log \frac{[E]}{[E] - [x]}$$

$$= \frac{2.303}{t[I]} \log \frac{100}{\% \text{ Activity}}$$

From the slope of inhibitor concentration versus log percent activity curves it is possible to calculate the kinetic constants of inhibition. Table 3 shows the inhibition rate constants (k_2) of bovine red cell, fly-head and mouse brain cholinesterases with Phosdrin and Bomyl isomers at 25°, 30° and 35°. The rate of the reaction increases with

increase in temperature. The energy of activation for the reaction between the cholinesterases and Phosdrin isomers was calculated from the data in Table 3, and found to be approximately 10Kcal/mol in all cases (60).

Another way of stating the effectiveness of cholinesterase inhibitors is the pI_{50} expression (= minus log of inhibitor concentration required for 50% inhibition). This expression is easily comprehended whereas the units of k_2, $1 \times min^{-1} \times moles^{-1}$, are not. Nevertheless, the pI_{50} expression has the disadvantage of being time dependent. Table 4 shows the inhibition of bovine red cell, mouse brain and fly-head cholinesterases by Phosdrin and Bomyl isomers expressed as pI_{50}. Fly-head cholinesterase is much more susceptible to inhibition than the bovine red cell and mouse brain enzymes. Cis Phosdrin is about one hundred times better as an inhibitor than the trans isomer, whereas both Bomyl isomers are equally good inhibitors.

b) Reactivation of Cholinesterases Inhibited by Phosdrin and Bomyl Isomers. Cholinesterase inhibited by organophosphates is a phosphorylated enzyme, probably a phosphoryl ester of a serine residue at the active site. Reactivation can occur by hydrolysis of this ester in a reaction that follows first-order kinetics.

Figure 8 shows the reactivation of bovine red cell, mouse brain, and fly-head cholinesterases inhibited by the isomers of Phosdrin and Bomyl. The different slopes indicate dissimilar reactivation rates.

FIGURE 7

Inhibition of cholinesterases by cis and trans Phosdrin at 25°.

Inhibition of bovine red cell cholinesterase by cis Phosdrin (Figure A) and trans Phosdrin (Figure B). When inhibitor concentration was varied the inhibition time was 30 min. When inhibition time was studied cis Phosdrin and trans Phosdrin concentrations were $1.8 \cdot 10^{-7}$M and $1.8 \cdot 10^{-6}$M respectively.

Inhibition of fly-head cholinesterase by cis Phosdrin (Figure C) and trans Phosdrin (Figure D). As in Figures A and B, the inhibition time was 30 min. when inhibitor concentration was varied. When inhibition time was studied, the inhibitor concentrations were $1.8 \cdot 10^{-8}$M (cis-Phosdrin) and $1.8 \cdot 10^{-6}$M (trans-Phosdrin).

General procedure as indicated under Determination of cholinesterase activity.

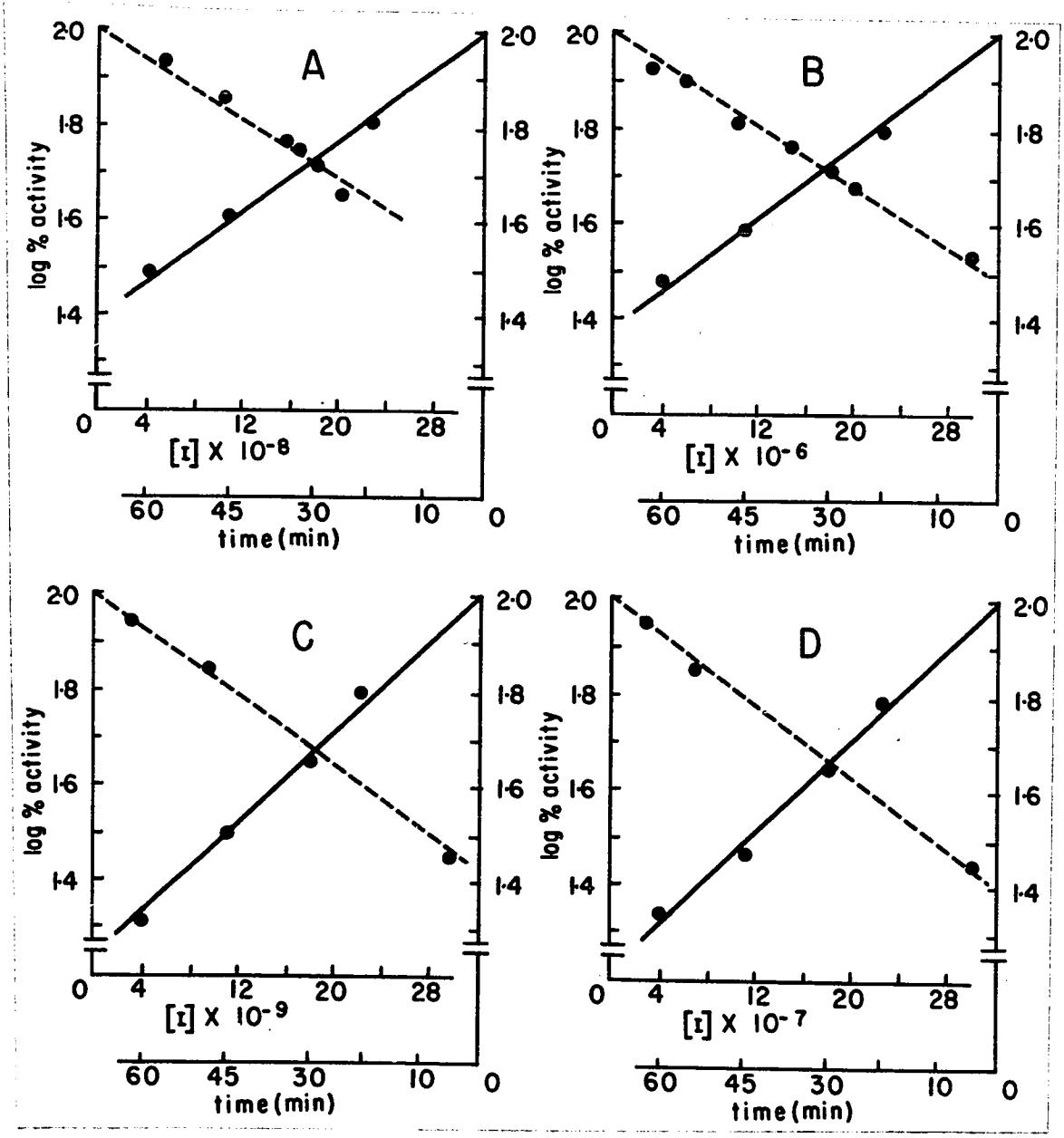

A
B
C
D
log % activity
2·0
1·8
1·6
1·4
0
4
12
20
28
[I] X 10⁻⁸
[I] X 10⁻⁶
[I] X 10⁻⁹
[I] X 10⁻⁷
60
45
30
10
0
time (min)

FIGURE 7

TABLE 3

INHIBITION OF CHOLINESTERASE BY PHOSDRIN AND BOMYL ISOMERS

Temperature	Enzyme Sources	k_2 (1/mol. min.)[1, 2] Cis Phosdrin	Trans Phosdrin	Cis Bomyl	Trans Bomyl
25°	Bovine red cell	1.3×10^5	1.3×10^3	7.6×10^5	7.3×10^5
	Mouse brain	3.4×10^5	4.1×10^3	13.0×10^5	14.0×10^5
	Fly head	14.0×10^5	14.0×10^3	73.0×10^5	73.0×10^5
30°	Bovine red cell	1.8×10^5	1.7×10^3		
	Mouse brain	----	----		
	Fly head	17.0×10^5	18.0×10^3		
35°	Bovine red cell	2.3×10^5	2.3×10^3		
	Mouse brain	5.6×10^5	6.2×10^3		
	Fly head	21.0×10^5	23.0×10^3		

[1] Inhibition rate constants calculated from slope of inhibitor concentration versus log percent activity curves (Inhibition time 30 min.). Because considerable reactivation of the enzymes occurred at 35°, the amount of carbon dioxide produced in the cholinesterase assay was extrapolated to zero time.

[2] General procedure as indicated under determination of cholinesterase activity.

TABLE 4

INHIBITION OF BOVINE RED CELL, MOUSE BRAIN AND FLY HEAD CHOLINESTERASE BY PHOSDRIN AND BOMYL ISOMERS

Enzyme source[2]	Phosdrin		Bomyl	
	cis	trans	cis	trans
	(Results as pI_{50} at 25°, 30')[1]			
Bovine red cell	6.75	4.75	7.52	7.50
Mouse brain	7.16	5.25	7.75	7.80
Fly head	7.77	5.77	8.50	8.50

[1] pI_{50} at 25°, 30' = minus log of concentration of vinyl organophosphage to produce 50% inhibition of the enzyme at 25° in 30 min.

[2] Bovine red cell enzyme, 0.240mg of commercial preparation per flask; mouse brain enzyme, the equivalent of 48mg of brain per flask; fly head enzyme, the equivalent of four fly heads per flask. Under the assay conditions control flasks evolved 110-130µl. CO_2 in 30 min.

The figure also shows that regardless of the dimethyl phosphate used to inhibit any one of the enzymes the rate of reactivation is approximately the same. Our results for the three enzymes are in agreement with those reported by Aldridge and Davison (45) for the reactivation of erythrocyte cholinesterase inhibited with different dimethyl phosphates.

3. DEGRADATION

a) Degradation of Phosdrin and Bomyl Isomers by Mouse Liver and Fly Homogenates. Carboxyester-containing organophosphorus cholinesterase inhibitors can be detoxified by cleavage of the carboxy or phosphate ester. The resulting negatively charged molecule is a poor inhibitor of cholinesterase because of the repulsion of the negative charge at the anionic site and a decreased electrophilic character of the phosphorus group.

Table 5 shows the degrading activity of mouse liver and fly homogenates on the isomers of Phosdrin and Bomyl. Fly homogenates showed no measurable degradation of the compounds but mouse liver was very active. Trans Phosdrin was degraded faster than the cis isomer; cis and trans Bomyl were degraded at approximately the same rate.

b) Degradation of Phosdrin and Bomyl isomers by mouse liver fractions. Centrifugal fractionation of a mouse liver homogenate and assay of the individual fractions (Table 6) shows that, for the Phosdrin isomers most of the degrading activity was found in the supernatant,

FIGURE 8

Reactivation of bovine red cell, mouse brain, and fly head cholinesterase inhibited by Phosdrin and Bomyl isomers.

Key:- o - o fly head enzyme

x - x mouse brain enzyme

o - o bovine red cell enzyme

Enzymes were incubated with the inhibitor for 30 min. at 35° before the substrate acetylcholine bromide was added. The molar concentrations of the organophosphorus compounds in the incubation mixture with bovine red cell, mouse brain, and fly head enzyme, respectively, were cis Phosdrin: 2×10^{-7}, 10^{-7}, and 2×10^{-8}; trans Phosdrin: 2×10^{-5}, 10^{-5}, and 2×10^{-6}; cis and trans Bomyl; 6.4×10^{-8}, 2×10^{-8}, and 6.4×10^{-9}.

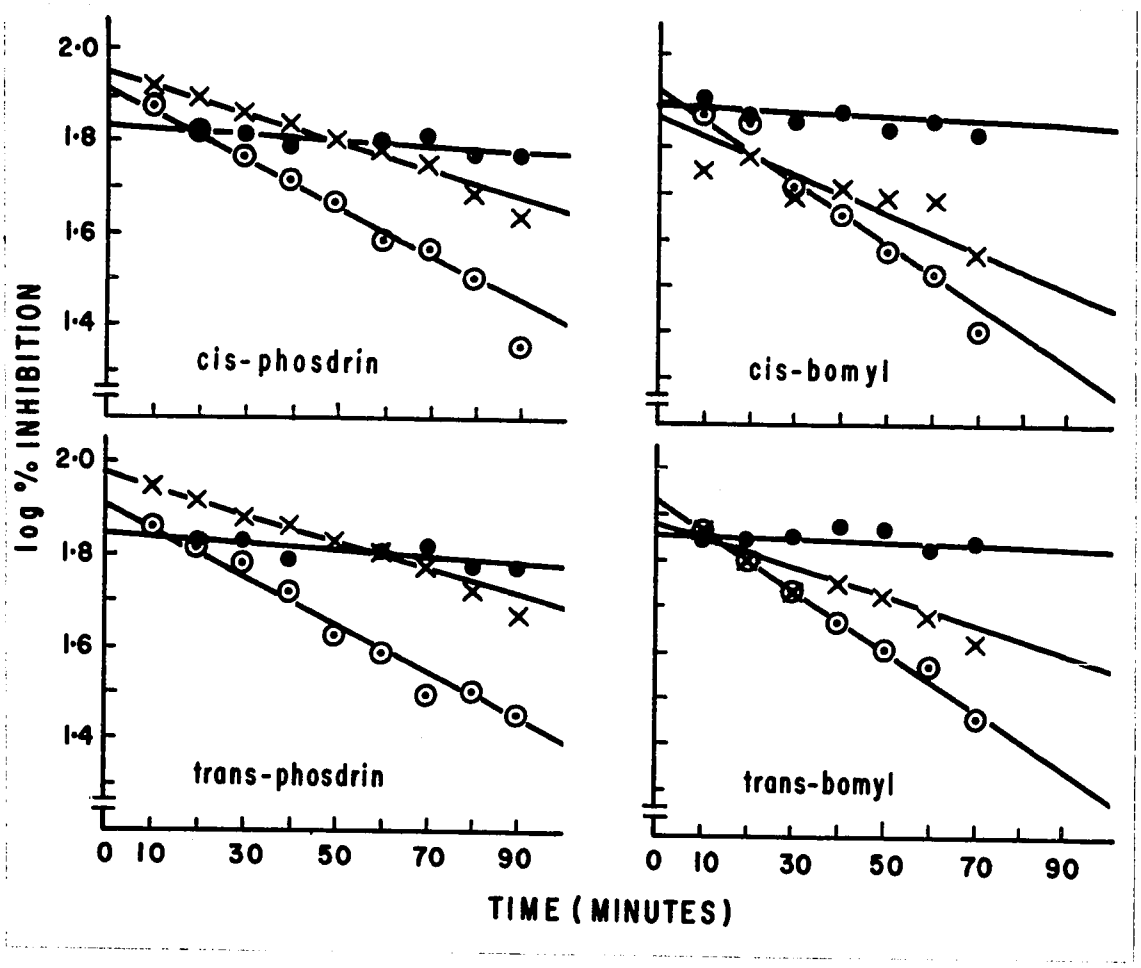

2·0
1·8
1·6
1·4
log % INHIBITION
cis-phosdrin
cis-bomyl
trans-phosdrin
trans-bomyl
0 10 30 50 70 90
TIME (MINUTES)

FIGURE 8

TABLE 5

DEGRADATION OF PHOSDRIN AND BOMYL ISOMERS BY MOUSE LIVER AND FLY HOMOGENATES

Organo-phosphate	Concentration[1] (M)	Incubation[2]	Detoxication[3] (%)
Cis Phosdrin	2.35×10^{-4}	Mouse liver	48
		Fly	0
		Control	0
Trans Phosdrin	2.35×10^{-4}	Mouse liver	80
		Fly	0
		Control	0
Cis Bomyl	3.2×10^{-4}	Mouse liver	77
		Fly	0
		Control	0
Trans Bomyl	3.2×10^{-4}	Mouse liver	80
		Fly	0
		Control	0

[1] Concentration of the vinyl organophosphate in the initial incubation mixture.

[2] Incubation in 0.0125M phosphate buffer (pH 7.5) at 35°. Mouse liver homogenate equivalent to 50mg. Fly homogenate equivalent to 20 flies. For Phosdrin isomers the incubation volume was 1.15ml and the incubation time 60 min; for Bomyl isomers, 1 and 30 respectively. At the end of the incubation appropriate dilutions were made and the mixtures were tested in the cholinesterase assay as explained in Methods.

[3] Calculated from bovine red cell inhibition curves (log percent inhibition versus inhibitor concentration). Controls represent incubations carried out with homogenates that were treated in a boiling water bath for 3 min.

TABLE 6

DEGRADATION OF PHOSDRIN AND BOMYL ISOMERS BY MOUSE LIVER FRACTIONS

Fraction[1,2]	% Detoxication[3]			
	Phosdrin		Bomyl	
	cis isomer	trans isomer	cis isomer	trans isomer
Homogenate	48	80	77	80
Microsomes	3	15	32	30
Supernatant	35	70	32	31

[1] Incubation in 0.0125M phosphate buffer (pH 7.5) at 35°C. Mouse liver fractions equivalent to 50mg. For Phosdrin isomers the concentration was 2.35×10^{-4}M, the incubation volume 1.15ml and the incubation time 60 min.; for Bomyl isomers, 3.2×10^{-4}, 1 and 30 respectively. At the end of the incubation appropriate dilutions were made and the mixtures were tested in the cholinesterase assay.

[2] The unwashed low speed (10,000g) precipitate contained only a small amount of activity.

[3] Calculated from cholinesterase inhibition curves. Control incubations with homogenates that were treated in boiling water for 3 min. showed no degradation of the organophosphates used.

whereas for Bomyl isomers activity was equally distributed between the supernatant and the microsomal fraction.

c) Effect of TOCP on the Toxicity of Phosdrin, Bomyl and Thiono Phosdrin Isomers to the Mouse. Malathion and malaoxon are insecticides of low toxicity to mammals, because of extensive degradation involving hydrolysis of their carboxyester groups. O'Brien (32, 33) showed that selectivity was more or less a general property of carboxyester-containing organophosphorus compounds. TOCP is known to be a good in vivo inhibitor of carboxyesterases and consequently potentiates the toxicity of carboxyester-containing organophosphorus compounds (19, 20, 46). Phosdrin and Bomyl isomers are carboxyester containing organophosphorus compounds, and they should be potentiated by TOCP if carboxyesterase plays a significant role in their degradation. Table 7 shows that TOCP failed to potentiate Phosdrin, Bomyl and thiono Phosdrin isomers to any significant extent, while malaoxon, under the same experimental conditions, was potentiated 24 fold.

d) The Role of Glutathione and Aging on the Phosdrin and Bomyl Degradation by Mouse Liver. Table 8 shows that mouse liver supernatant lost 75% of its cis Phosdrin degrading activity on dialysis, and that the addition of reduced glutathione fully restored the original activity. Trans Phosdrin detoxication was not affected to any considerable extent by dialysis or the addition of reduced glutathione. The small amount of cis Phosdrin degrading activity in the dialized supernatant is probably

TABLE 7

EFFECT OF TOCP ON THE TOXICITY OF PHOSDRIN, BOMYL AND THIONO-PHOSDRIN ISOMERS TO THE MOUSE

Compound	LD_{50} (mg/Kg)[1]		Ratio
	----	after pretreatment with TOCP[2]	LD_{50}/LD_{50} (TOCP)
Cis Phosdrin	2	1.2	1.7
Trans Phosdrin	45	30.0	1.5
Cis Bomyl	1.6	0.55	2.9
Trans Bomyl	1.4	0.47	3.0
Cis Thiono Phosdrin	43	14.5	3.0
Trans Thiono Phosdrin	930	830	1.1
Malaoxon	118	4.9	24.0

[1] Intraperitoneal injection in 0.2ml of water for phosphates or 0.2ml of oil for thiono phosphates.

[2] TOCP intraperitoneally in 0.2ml of oil at a dose of 400mg/Kg, 4 hours before the toxicity determination.

TABLE 8

THE ROLE OF GLUTATHIONE AND AGING IN THE DEGRADATION OF PHOSDRIN AND BOMYL BY MOUSE LIVER

Liver Supernatant[1]	% Detoxication[2]	
	Cis Phosdrin	Trans Phosdrin
Control[3]	94	88
Aged[4]	94	91
Dialized	24	81
Dialized + Reduced Glutathione	96	90
	Cis Bomyl	Trans Bomyl
Control[3]	70	75
Aged[4]	47	50
Dialized	28	35
Dialized + Reduced Glutathione	35	41

[1] Incubations in 15mM phosphate buffer (pH 7.5) for 15 min at 35°. Mouse liver supernatant equivalent to 100mg of liver. Phosdrin and Bomyl 0.3mM; reduced glutathione (when added) 4mM. Incubation volume 1ml. Controls without supernatant or supernatant boiled for 3 min. showed no degradation of the organophosphate used.

[2] Calculated from cholinesterase inhibition curves.

[3] Liver supernatant assayed immediately after preparation.

[4] Liver supernatant assayed 6 hours after preparation, comparable to the dialized supernatant (see Methods).

due to glutathione remaining after dialysis (e.g. protein bound). Cis and trans Bomyl degradation was not affected to any considerable extent by the presence or absence of glutathione. The degrading activity toward Phosdrin isomers was stable since no irreversible loss occurred during standing (6 hrs at 3°) with or without dialysis. By contrast, about half of the degrading activity toward Bomyl isomers was lost under the same experimental conditions.

e) Effect of Age of the Animals on Phosdrin Degradation by Mouse Liver. The capacity of liver supernatants prepared from "young" mice to degrade cis Phosdrin is about half that of supernatants prepared from old mice (Table 9). This difference is probably not due to different enzyme concentrations, but rather to a lower glutathione content of livers from young mice, since on the addition of reduced glutathione their activities become approximately equal. Trans Phosdrin degradation was not affected by the age of the mice, or the addition of glutathione (Table 9).

f) Identification of the Glutathione Metabolite. Glutathione and methyl glutathione have very similar chromatographic mobility, but can be resolved after treatment with iodoacetamide (43). The "product of the reaction" shows one spot at R_f 0.31 which after treatment with iodoacetamide is resolved to two spots of similar intensity with R_f values of 0.31 and 0.16, corresponding to methyl glutathione and glutathione respectively (Table 10). It should be noted that glutathione was added

TABLE 9

EFFECT OF AGE OF THE ANIMALS ON PHOSDRIN DEGRADATION BY MOUSE LIVER

Mice	System [1]	% Detoxication [2] Cis Phosdrin	Trans Phosdrin
Young (18g live weight)	Liver supernatant	42	77
	Liver supernatant + Reduced Glutathione	88	79
Old (35g live weight)	Liver supernatant	87	81
	Liver supernatant + Reduced Glutathione	90	79

[1] Incubation in 15mM phosphate buffer (pH 7.5) for 15 min. at 35°. Mouse liver supernatant equivalent to 50mg liver. Phosdrin 0.3 mM, reduced glutathione (when added) 4mM. Incubation volume was 1ml. Control incubations carried out without supernatants or supernatants that were treated in a boiling water bath for 3 min. showed no degradation of the organophosphate used.

[2] Calculated from cholinesterase inhibition curves.

TABLE 10

IDENTIFICATION OF THE GLUTATHIONE METABOLITE

Compounds	R_f [1]	R_f (After treatment with Iodoacetamide)	
Standard reduced glutathione	0.32		0.19
Standard methyl-glutathione	0.37	0.34	
Standard reduced glutathione + standard methyl-glutathione	0.35	0.32	0.18
Product of the reaction	0.31	0.31	0.16

[1] Ascending chromatography on Whatman No. 1 paper. Solvent mixture: butan-1-ol:acetic acid:water:mercaptoethanol (11:4±5:0.1). Chromatograms were sprayed with ninhydrin reagent. Standards 50µg per chromatogram; product of the reaction equivalent to 100µg of reduced glutathione in the incubation mixture. See Methods for incubation procedure.

in approximately 100% excess with respect to cis Phosdrin, assuming a reaction molar ratio of 1:1. A spot of R_f 0.1 was noted in chromatograms with standard glutathione, as well as with "product of the reaction." This spot was greatly diminished by the use of mercaptoethanol in the solvent system and probably corresponds to oxidized glutathione.

g) Chromatographic identification of organophosphate metabolites.

Phosdrin and Bomyl Isomers: Acid Procedure

Chromatography of the chloroform extracts from incubation mixtures containing Phosdrin or Bomyl isomers (see Methods) showed no phosphorus containing compounds, except for a small amount of unreacted organophosphates (R_f = 0.90 - 0.95). These chromatograms indicated that there was no detectable carboxyesterase activity, since the expected products would be extracted into chloroform from an aqueous phase at low pH (47).

Table 11 shows that the metabolites from trans Phosdrin, cis Bomyl and trans Bomyl have R_f values corresponding to standard dimethyl phosphate. Co-chromatography of these metabolites with dimethyl phosphate resulted in a single spot. Dimethyl phosphate was found to be stable under the incubation and purification conditions.

The cis Phosdrin metabolite has R_f values of 0.64 in system A and 0.09 in system B. Monomethyl phosphate exhibits the same R_f

TABLE 11

CHROMATOGRAPHIC IDENTIFICATION OF PHOSDRIN AND BOMYL METABOLITES : ACID PROCEDURE[1]

Phosphorus-Containing Metabolite from:	Standards	R_f Values[2] System A	System B	
Trans Phosdrin	---	0.70	0.49	
Cis Bomyl	---	0.70	0.49	
Trans-Bomyl	---	0.70	0.49	
---	Dimethyl phosphate	0.72	0.49	
[3]Trans Phosdrin	Dimethyl phosphate	0.70	0.49	
[3]Cis Bomyl	Dimethyl phosphate	0.70	0.49	
[3]Trans Bomyl	Dimethyl phosphate	0.70	0.49	
Cis Phosdrin	---	0.64	0.09	
---	Monomethyl phosphate	0.68	0.10	
---	Cis desmethyl Phosdrin Na^+	0.65	0.62	
---	Cis desmethyl Phosdrin Na^+ (acid treated)[4]	0.68	0.09	
[3]Cis Phosdrin	Cis desmethyl Phosdrin Na^+	0.64	0.09	0.62
[3]Cis Phosdrin	Monomethyl phosphate	0.68	0.09	
[3]Cis Phosdrin	Cis desmethyl Phosdrin Na^+ (acid treated)[4]	0.68	0.09	
[3]Monomethyl phosphate + Cis desmethyl Phosdrin Na^+ (acid treated)[4]		0.68	0.09	

[1] See Methods for incubation and purification procedures.

[2] Ascending chromatography on Whatman No. 1 paper. System A: butan-1-ol:acetic acid:water (11:4:5). System B: Isopropanol: NH_3 (d = 0.88) (2.5:1). Standards and metabolites (assuming 100% recovery) 200µg per chromatogram.

[3] Indicates co-chromatography.

[4] Cis-desmethyl Phosdrin Na^+ chemically prepared and subjected to the same purification procedure as the cis-Phosdrin metabolite (see Materials and Methods).

values and co-chromatography with the metabolite resulted in a single spot. These results would indicate that the cis Phosdrin metabolite is monomethyl phosphate. However, when standard cis desmethyl Phosdrin Na^+ was treated as the cis Phosdrin metabolite, it changed to a compound of similar R_f value to monomethyl phosphate, which upon co-chromatography with monomethyl phosphate produced a single spot. These findings indicate that the cis Phosdrin metabolite is cis desmethyl Phosdrin, which during the extraction and purification procedure is hydrolyzed to monomethyl phosphate.

Cis Phosdrin: Neutral Procedure

Table 12 shows that cis Phosdrin metabolite purified at neutral pH (see Methods), has R_f values, in system A and B, similar to those of standard cis-desmethyl Phosdrin Na^+. Co-chromatography of the metabolite with cis desmethyl Phosdrin produces a single spot. Table 12 also shows that cis desmethyl Phosdrin Na^+ was not further metabolized, and that it was stable under the procedures of extraction and purification. These results add further support to the conclusion that the major metabolite from cis Phosdrin is cis desmethyl Phosdrin.

4. ACTIVATION OF THIONO PHOSDRIN ISOMERS

a) Metabolism of Thiono Phosdrin Isomers by Mouse Liver and Fly Slices. Table 13 shows that cis thiono Phosdrin was activated by mouse liver and fly slices to the same extent, while no measurable activation of the trans isomer was observed.

TABLE 12

CHROMATOGRAPHIC IDENTIFICATION OF CIS PHOSDRIN METABOLITE NEUTRAL PROCEDURE[1]

Phosphorus-containing Metabolite from:	Standards	R_f Values[2] System A	System B
Cis Phosdrin	---	0.61	0.54
[3]Cis Phosdrin	Cis desmethyl Phosdrin Na^+	0.61	0.54
---	Cis desmethyl Phosdrin Na^+	0.65	0.63
Cis desmethyl Phosdrin Na^+	---	0.61	0.54

[1] See Methods for incubation and purification procedures.

[2] Ascending chromatography on Whatman No. 1 paper. System A: butan-1-ol:acetic acid:water (11:4:5). System B: Isopropanol: NH_3 (d = 0.88) (2.4:1). Standards and metabolites (assuming 100% recovery) 200µg per chromatogram.

[3] Indicates co-chromatography.

TABLE 13

METABOLISM OF THIONO PHOSDRIN ISOMERS BY MOUSE LIVER AND FLY SLICES

Animal	Weight of Tissue Added (mg)	Oxygen analog at the end of incubation[1,2] (mμ moles/hr/g wet tissue) Cis isomer	Trans isomer
Mouse	100	70	0
	200	65	-2
Fly	400	62	1

[1] Calculated from cholinesterase inhibition curves.

[2] Incubations were carried out for 1 hr at 37° in a total volume of 2ml containing: phosphate buffer (pH 7.5), 12.4×10^{-3}M; thiono Phosdrin, 4.5×10^{-3}M; mouse liver or fly slices. After incubation an aliquot was tested against cholinesterase (see Methods).

In attempting to evaluate these results it should be kept in mind that since mouse liver slices and homogenates are known to degrade Phosdrin isomers (Tables 5 and 15), the present experiments, at least in the case of mouse liver, reflect the balance between activation of thiono Phosdrin to produce the potent cholinesterase inhibitor Phosdrin, and degradation of the latter.

b) Metabolism of Thiono Phosdrin Isomers by Mouse Liver and Fly Homogenates and Microsomes. Table 14 shows that trans thiono Phosdrin was not activated by mouse liver or fly homogenates or microsomes. Even when mouse liver preparations equivalent to 1g of tissue were used, no measurable activation of trans thiono Phosdrin was found. The cis isomer was activated approximately to the same extent by mouse liver homogenates and microsomes. The latter observation is unexpected because mouse liver homogenates are known to degrade Phosdrin isomers very actively (Tables 5 and 6) while microsomes do not (Table 5). A likely explanation of these results is offered in the Discussion. $NADPH_2$ was found to be a little better than $NADH_2$ in supporting the enzymatic oxidation of cis thiono Phosdrin.

Fly homogenates and microsomes were unable to activate cis thiono Phosdrin to any significant extent, indicating that the activity present in the slices was lost upon homogenization of the tissue.

The presence of microsomal inhibitors in insect preparations has been postulated (58). In order to investigate the presence of an

TABLE 14

METABOLISM OF THIONO PHOSDRIN ISOMERS BY MOUSE LIVER AND FLY HOMOGENATES AND MICROSOMES

Tissue	Reduced pyridine nucleotide added	Oxygen analog at the end of incubation[1] (mμ moles/hr/g wet tissue)			
		Homogenates[2]		Microsomes[3]	
		cis isomer	trans isomer	cis isomer	trans isomer
Mouse liver	$NADH_2$	70	-3	105	-2
	$NADPH_2$	120	2	120	0
Fly	$NADH_2$	-1	0	0	0
	$NADPH_2$	2	-2	0	-2

[1] Calculated from cholinesterase inhibition curves.

[2] Incubations were carried out for 1 hr at 37° in a total volume of 1.15ml containing: phosphate buffer (pH 7.5), 12.5×10^{-3}M; thiono Phosdrin, 3.9×10^{-3}M; $NADH_2$ or $NADPH_2$, 4×10^{-4}M; mouse liver homogenate equivalent to 50mg wet tissue; fly homogenate equivalent to 200mg (approximately 10 decapitated flies). After incubation an aliquot was tested against cholinesterase (see Methods).

[3] Same conditions as incubation with homogenates[2] except for: mouse liver microsomes equivalent to 250mg wet tissue; fly microsomes equivalent to 1g of decapitated flies; $MgCl_2$ 13×10^{-3}M.

inhibitor or some unknown cofactor required for the fly microsomal preparations, mixed microsomes (fly and mouse liver) or a microsomal fraction prepared from mixed homogenates (fly and mouse liver) were tested for cis thiono Phosdrin activation. The activity of these preparations was found to be equal to that of mouse liver microsomes alone.

c) Protective Effect of Thiono Phosdrin Isomers on the Degradation of Phosdrin Isomers by Mouse Liver Slices. Table 15 shows that trans Phosdrin was more extensively degraded by mouse liver slices than the cis isomer. Both isomers were protected from degradation by their respective thiono analog, the cis isomer showing the largest effect. Strictly speaking these experiments represent a balance between degradation of the phosphates and activation of the added phosphorothionates. However, the magnitude of the observed protection in terms of mμmoles of Phosdrin could not possibly be explained on the basis of the much less efficient activation process (Tables 13 and 14).

Fly slices (200mg wet tissue) under the experimental conditions of Table 15 were unable to degrade Phosdrin isomers to any significant extent.

d) Factors Influencing cis Thiono Phosdrin Activation by Mouse Liver Microsomes. Table 16 shows that the activation of cis thiono Phosdrin by mouse liver microsomes is enzymatic and requires molecular oxygen and reduced NADP. The enzymatic system must have a very high affinity for oxygen as judged from the severe conditions

TABLE 15

PROTECTIVE EFFECT OF THIONO PHOSDRIN ISOMERS ON THE DEGRADATION OF PHOSDRIN ISOMERS BY MOUSE LIVER SLICES

Incubation[1]	mμ moles of Phosdrin isomers degraded per hour per g of wet tissue[2]
Cis Phosdrin	2,100
Cis Phosdrin + Cis thiono Phosdrin	360
Trans Phosdrin	3,150
Trans Phosdrin + Trans thiono Phosdrin	2,000

[1] Incubations were carried out for 1 hr. at 37° in a total volume of 2ml containing: phosphate buffer (pH 7.5) 12.5×10^{-3}M; Phosdrin 1.8×10^{-4}M; thiono Phosdrin (when added) 1.5×10^{-3}M; mouse liver slices 100mg. After incubation an aliquot was tested against cholinesterase (see Methods).

[2] Calculated from cholinesterase inhibition curves.

TABLE 16

FACTORS INFLUENCING CIS THIONO PHOSDRIN ACTIVATION BY MOUSE LIVER MICROSOMES

System[1]	Activation (% complete system)
Complete[2]	100
----- minus microsomes	1
----- minus microsomes (boiled microsomes added)	3
----- minus Oxygen[3]	22
----- minus Mg^{++}	100
----- minus Nicotinamide	105
----- minus $NADPH_2$	-4

[1] Incubations were carried out for 1 hr at 37° in a total volume of 1.15ml. Complete system contained: phosphate buffer (pH 7.5), 12.5×10^{-3}M; thiono Phosdrin, 3.9×10^{-3}M; $NADPH_2$, 4×10^{-4}M; $MgCl_2$, 13×10^{-3}M; nicotinamide, 13×10^{-3}M; microsomes from 250mg of liver (wet tissue). After incubation an aliquot was tested against cholinesterase (see Methods).

[2] Complete system produced 114mμ moles of oxygen analog per hour per g of wet tissue.

[3] Before and during incubation the flasks were gassed extensively with nitrogen that had been bubbled through Fieser's solution (to trap small amount of oxygen present in commercial nitrogen). When nitrogen was used directly from the cylinder, 100% activation was obtained.

necessary to eliminate oxygen, and consequently shows its requirement. Mg^{++} and nicotinamide showed no effect on the system. Mg^{++} is known to be present in microsomal preparations (48). When $NADH_2$ was used instead of $NADPH_2$ the system was found to be more sensitive to the lack of oxygen and Mg^{++}, the percent activity with respect to the complete system being 6 and 68 respectively.

DISCUSSION AND CONCLUSIONS

1. INHIBITION AND REACTIVATION OF CHOLINESTERASES

Cholinesterases from several sources show different susceptibility to organophosphorus inhibitors (Table 4). O'Brien (49) proposed that cholinesterases vary with respect to the distance between the anionic and esteratic sites. The effectiveness of an organophosphorus inhibitor depends partly on the electrophilic character of the phosphorus group (that interacts with the esteratic site) and also on the presence of a group that interacts electrostatically or through noncoulombic forces (e.g. Van der Waals, or hydrophobic) with the anionic site (59a, 59b, 59c). For ideal binding, the latter group should be at a distance from the phosphorus equal to the distance between the anionic and esteratic sites of the enzyme. Possibly, for Phosdrin and Bomyl isomers the interaction of a methyl carboxyester group with the anionic site of cholinesterases plays an important role in binding. The distance between the methyl carboxyester group(s) of Bomyl isomers and of cis Phosdrin and their respective phosphate groups is approximately the same (Table 17) and is in the range of distance (4.5-5.9 $\mathring{A}$) reported to exist between the anionic and the esteratic site of insect cholinesterase (49). In the case of trans Phosdrin, this distance is much smaller and consequently, this compound is a poorer inhibitor of insect cholinesterase

TABLE 17

DISTANCES BETWEEN THE PHOSPHATE AND THE CARBOXYESTER GROUPS OF PHOSDRIN AND BOMYL[1]

	Phosdrin		Bomyl	
	cis	trans	cis	trans
Range of distances	4.3-5.2	2.2-4.4	4.4-5.2	4.4-5.2
Average	4.75	3.3	4.8	4.8

[1] Distances (Å) given are between the centres of the phosphorous atom and the carbonyl carbon. At any possible configuration the molecules have the carbonyl carbon (Phosdrin) or at least one of the carbonyl carbons (Bomyl) in the range of distances shown.

than cis Phosdrin or the Bomyl isomers.

According to evidence presented by Main (50), the reaction between organophosphates and cholinesterases probably involves a reversible step which precedes phosphorylation and results in the formation of an enzyme-inhibitor complex. The bimolecular rate constants reported here (Table 3) may therefore involve both a phosphorylation and an affinity constant.

The rate of aqueous reactivation of a given cholinesterase (bovine red cell, mouse brain or fly head) is approximately the same regardless of the organophosphate used to inhibit it (slopes of curves in Figure 8). This fact further supports the idea that the inhibited enzyme is a phosphorylated one, since Bomyl and Phosdrin isomers are dimethyl phosphates that would be expected to produce the same inhibited enzyme (i.e. dimethyl phosphoryl-E).

2. DEGRADATION OF PHOSDRIN AND BOMYL ISOMERS

It is possible to visualize that Phosdrin and Bomyl isomers could be hydrolytically degraded at the P-O-C=C, P-O-CH_3 or carboxyester group(s) (see Figure 2). Hydrolysis at the methyl carboxyester group(s) does not seem to play an important role in detoxication of Bomyl and Phosdrin isomers since TOCP, a known carboxyesterase inhibitor, failed to potentiate their toxicity (Table 7). Also, no carboxyesterase metabolites could be detected.

Resistance to malathion is usually due to increased levels of

carboxyesterase. Recently van den Heuvel and Cochran (51) reported that Blattella germanica resistant to malathion exhibits no cross-resistance with Bomyl (referred in their paper as GC-3707). These findings were unexpected because of the close structural similarity between Bomyl and malathion. The results presented in this communication indicating that carboxyesterase is not involved in the degradation of Bomyl offers an explanation of the findings of the above authors.

Cis Phosdrin is degraded by mouse liver homogenates via a reduced-glutathione S-alkyl transferase. This conclusion is based on the finding that the reaction is reduced glutathione dependent (Table 8) and on the chromatographic identification of the products as methyl glutathione (Table 10) and cis desmethyl Phosdrin (Tables 11 and 12).

Trans Phosdrin and both Bomyl isomers are mainly degraded by cleavage at the P-O-C=C group, the phosphorus-containing metabolite being dimethyl phosphate (Table 11). Our results show also that the enzymatic system(s) responsible for the degradation of trans Phosdrin and those responsible for degradation of the Bomyl isomers are different, because of the observed differences in cellular localization and stability (Tables 6 and 8).

As shown in Table 5, Phosdrin and Bomyl isomers were not degraded to any measurable extent by fly-homogenates. We repeated these experiments adding reduced-glutathione to the incubation mixtures and found no change. Fly supernatants (100,000 x g) or fly microsomes

also were found unable to degrade Phosdrin and Bomyl isomers to any measurable extent.

Tables 5 and 6 show that mouse liver homogenates degraded the trans isomer of Phosdrin to a greater extent than the cis. The results presented in Tables 8 and 9 indicate that availability of reduced-glutathione is essential in the in vitro degradation of cis Phosdrin. However, the glutathione level in liver and other mammalian tissues is such that it would not likely be a limiting factor in the in vivo detoxification of cis Phosdrin.

Probably cis and trans thiono Phosdrin are degraded in a way similar to their respective oxygen analogs because: a) TOCP failed to potentiate their toxicity (Table 7); and b) thiono Phosdrin isomers were found to protect the Phosdrin isomers against degradation (Table 15).

The present results, offer an interesting example of marked differences in the biological properties of a compound, as a consequence of geometrical isomerism.

The importance of glutathione-dependent enzymatic detoxication of organophosphorus compounds has recently become evident. Methyl parathion and Sumithion (both dimethyl phosphorus esters) have been reported to be metabolized to their respective desmethylated derivatives by a glutathione-dependent preparation (52), whereas the same preparation degrades ethyl parathion very slowly. Methyl iodide is degraded by rat liver in a similar way (43, 53). Our results show that while cis

Phosdrin is mainly degraded by a glutathione-dependent system, trans Phosdrin and both Bomyl isomers are not.

It is perhaps premature to generalize with regard to the stereospecificity of the glutathione-dependent enzyme system because the number of compounds studied till now is relatively small. Nevertheless, it is striking that compounds degraded to their respective desmethylated derivatives (e.g. methyl parathion, Sumithion, cis Phosdrin, methyl iodide) have their methyl groups relatively free. On the other hand, some dimethyl phosphates (e.g. trans-Phosdrin, cis and trans Bomyl) whose methyl groups are partially covered (by carboxyester group(s) in the examples above) are not degraded via demethylation to any significant extent.

The in vitro and/or in vivo metabolism of several dimethyl phosphorus compounds has been shown to produce, in variable proportions, metabolites resulting from cleavage at the P-O-CH_3 group. From the considerations outlined below it is possible that glutathione was involved in these degradations.

Hodgson and Casida (54) studied the metabolism of DDVP (a dimethyl phosphate) by rat liver homogenates and showed that the degradation products were of two kinds: P-O-CH_3 cleavage (demethylation) and P-O-CH=CCl_2 cleavage. From the point of view of the possible role of glutathione in DDVP degradation two sets of their data are very interesting: a) the study of the effect of metal ions on DDVP degradation

showed that dialysis reduced the degrading activity extensively and although the addition of certain metal ions stimulated degradation (through cleavage of DDVP at the P-O-vinyl group), the activity was far from being fully restored. This fact could be accounted for by the elimination of glutathione by dialysis. b) The effect of substrate concentration on DDVP degradation showed a large shift from P-O-CH_3 to P-O-$CH{=}CCl_2$ cleavage upon increase of DDVP concentration. When DDVP concentrations in the incubation mixture were 5×10^{-4}M and 2×10^{-2}M, the P-O-CH_3 cleavage products were approximately 1.7×10^{-4}M and 3.4×10^{-4}M and those of P-O-$CH{=}CCl_2$ cleavage 3.3×10^{-4}M and 70×10^{-4}M respectively (calculated from the data in ref 54). Johnson (43) has reported that rat liver contains approximately 5.2 μmoles of glutathione/g wet weight, while Fukami and Shishido (52) gave a value of 9.3μmoles/g. If glutathione concentration under Hodgson and Casida's (54) experimental conditions is calculated on the basis of the above values, it should be 2.1×10^{-4}M to 3.7×10^{-4}M. It is striking that glutathione concentration is in the range of the P-O-CH_3 cleavage products and that on increase of DDVP concentration in the incubation mixture, the increase in degradation is mainly of the P-O-vinyl type. This would indicate that glutathione availability is the limiting factor in the P-O-CH_3 cleavage.

Considerations similar to those above suggest the involvement of glutathione in Dipterex (a dimethyl phosphonate) degradation. Hassan

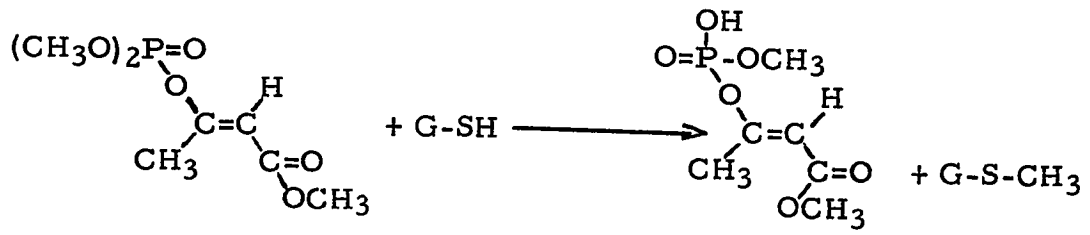
and Zayed (55) and Hassan et al (56) showed that Dipterex is partially degraded in vitro by cleavage of the P-O-CH_3 group. Injection of labelled Dipterex ($C^{14}H_3$) to the rat showed that radioactivity was eliminated in the expired air as $C^{14}O_2$ (55). The appearance of $C^{14}O_2$ was interpreted as a step by step oxidation of $C^{14}H_3OH$ (produced by P-O-CH_3 cleavage). Nevertheless, if glutathione plays a role, the methyl group would be transferred to glutathione producing S-$C^{14}H_3$-glutathione which is known to be degraded to S-methyl cysteine (43). The S-methyl group of the latter has been shown to be metabolized to CO_2 (57).

A summary of the proposed degradation of Phosdrin and Bomyl isomers by mouse liver is presented in Figure 9.

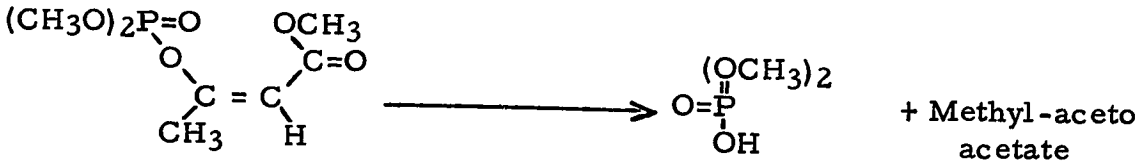
3. ACTIVATION OF THIONO PHOSDRIN ISOMERS

These results illustrate some of the factors contributing to the differences in toxicity of thiono Phosdrin isomers to the fly and the mouse. Thiono Phosdrin isomers are very poor cholinesterase inhibitors, approximately 5,000 times less effective than their respective oxygen analogs. Probably the small anticholinesterase activity of thiono Phosdrin is due to a slight contamination with the water soluble oxygen analog, Phosdrin. We have found that when thiono Phosdrin isomers were suspended in water with the aid of a detergent and the suspension was broken by high-speed centrifugation, almost all the anticholinesterase activity was found in the supernatant water phase.

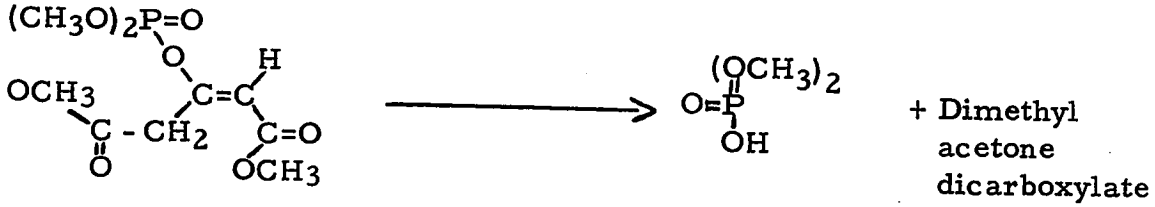
Figure 9: Proposed Degradation of Phosdrin and Bomyl Isomers by Mouse Liver



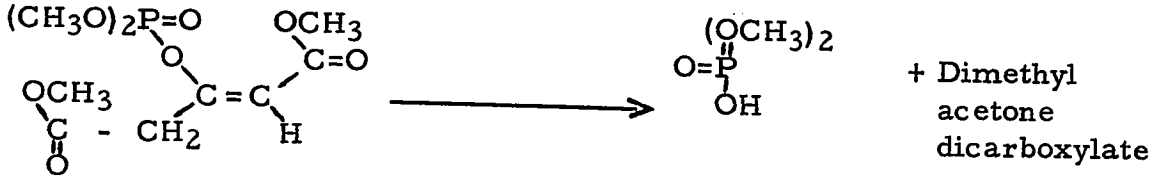
Cis-Phosdrin Glutathione Cis-desmethyl Phosdrin Methyl glutathione



Trans Phosdrin Dimethyl phosphate



Cis Bomyl Dimethyl phosphate



Trans Bomyl Dimethyl phosphate

Thiono Phosdrin isomers being poor inhibitors of cholinesterase probably are not toxic "per se" but rather because they can be oxidized to their respective oxygen analogs (Tables 13 and 14).

A comparison between Tables 13 and 14 shows that fly preparations lose their activating capacity of cis thiono Phosdrin on homogenization. The activation by mixed microsomal preparations (fly and mouse liver) was found to be equal to that of mouse liver microsomes alone. These experiments indicate a marked difference in stability between the fly and mouse microsomal fraction, rather than the presence of an inhibitor in the fly preparations.

Mouse liver homogenates, supernatants and slices degrade cis Phosdrin at approximately 2μmoles/hr/g wet tissue, while trans Phosdrin is degraded at about 3μmoles/hr/g (Tables 5, 6 and 15). The microsomal fraction (containing most of the activation enzyme(s)) activates cis thiono Phosdrin at a rate of approximately 0.1μmoles/hr/g (Table 14). Therefore the rate of degradation is much higher than that of activation. Nevertheless, accumulation of the oxygen analog occurs when cis thiono Phosdrin is incubated with mouse liver homogenates or slices (Tables 13 and 14). This apparent contradiction can be explained if thiono phosdrin protects the newly formed oxygen analog from degradation. Thiono Phosdrin was indeed shown to reduce the degradation of Phosdrin added to the incubation mixture, with the cis isomer exhibiting a greater protective effect than the trans (Table 15). It is possible that this effect is due to substrate competition.

Trans Phosdrin has been shown to be degraded mainly to dimethyl phosphate (Table 11). On the other hand, the cis isomer is cleaved to its respective desmethyl derivative in a reduced glutathione-dependent reaction (Tables 10, 11 and 12). The protective effect shown by cis thiono Phosdrin in cis Phosdrin degradation could possibly be due to a competition for the available glutathione.

In the experiments of Table 14 it is evident that the homogenate and the microsomal fraction activated cis thiono Phosdrin at approximately the same rate, in spite of the fact that microsomes as opposed to the whole homogenate do not contain any degrading activity (Table 6). An explanation can be found if the magnitude of the protective effect of thiono Phosdrin is realized. It should be pointed out that in the experiments shown in Table 15 the ratio thiono-Phosdrin/Phosdrin was approximately 10, while in the activation experiments (Tables 13 and 14) this ratio was at least 500.

Attempts to demonstrate activation of cis thiono Bomyl by mouse liver microsomes were unsuccessful, probably because microsomes degrade cis Bomyl very effectively (Table 6).

4. BIOCHEMICAL MECHANISMS AND TOXICITY

The present work presents evidence contributing to the understanding of the mode of action and consequently of the toxicity of Phosdrin, Bomyl, thiono Phosdrin and thiono Bomyl isomers.

Cis Phosdrin is more toxic than the trans isomer (Table 1),

possibly because of its stronger inhibition of cholinesterases (Table 4) and also, in the case of the mouse because of the somewhat slower degradation of the cis isomer by liver homogenates (Table 5).

Cis Phosdrin is more toxic to the fly than to the mouse (Table 1). This selectivity can be understood on the basis of the following considerations with regard to this particular organophosphate: a) mouse liver homogenates degrade it, whereas fly homogenates show no degrading activity (Table 5); b) inhibited mouse brain cholinesterase is reactivated much faster than the fly head enzyme (Figure 8); c) fly head cholinesterase is four to five times more susceptible to inhibition than mouse brain cholinesterase (Tables 3 and 4).

Both Bomyl isomers, by contrast with Phosdrin, are equally toxic (Table 1) probably because of their similarities in anticholinesterase activity and degradation rate (Tables 3, 4, 5 and 6).

The comparative in vitro properties of trans Phosdrin with fly and mouse tissue preparations are similar to those of the cis isomer (Tables 3, 4, 5 and Figure 8). The toxicological data reported by Spencer (37) where trans Phosdrin was found to be more toxic to the fly than to the mouse would be in good agreement with the biochemical data presented here. However, our toxicity data (Table 1) show that trans Phosdrin is equally toxic to the fly and the mouse. We do not have a satisfactory explanation for this discrepancy but it could be a result of penetration problems involved in the determination of toxicity to insects.

These problems are discussed at some length below.

Bomyl isomers exhibit similar toxicity toward the fly and the mouse (Table 1), in spite of the findings that both isomers are degraded more rapidly by mouse liver homogenates than by fly homogenates (Table 5) and that fly head cholinesterase is more susceptible to inhibition than mouse brain enzyme (Tables 3 and 4).

The LD_{50} values of Bomyl isomers to the fly are unexpectedly high, compared with that of cis Phosdrin (Table 1), considering that Bomyl isomers appear to be better inhibitors of fly head cholinesterase. These high LD_{50} values of trans Phosdrin and both Bomyl isomers obtained with the fly, as compared to those with the mouse, are possibly due to a slow penetration through the insect cuticle. The possibility that penetration in the insect is a more important factor than intraperitoneal absorption in the mouse, is supported by the following observations: a) The volume of the intraperitoneal injection had little influence on the LD_{50} values (range 0.05ml to 0.5ml). By contrast, in the case of the fly an LD_{50} value became an LD_{80} just by increasing the acetone volume (solvent used for topical application) from 1µl to 2µl. b) The toxicological data reported for thiono Phosdrin isomers with the mouse (Table 2) are very similar to those reported by Spencer (37), although in the first case the vehicle used was corn oil, and in the second a water emulsion. c) Unexpectedly, trans thiono Phosdrin is more toxic to the fly than trans Phosdrin (Tables 1 and 2) in spite of the fact that the latter

is the active anticholinesterase agent. But trans thiono Phosdrin is the more lipophilic of the two and can possibly penetrate the insect cuticle faster.

Cis thiono Phosdrin is more toxic than the trans isomer probably because of a higher activation rate (Table 13) and also because cis Phosdrin is approximately 100 times better as a cholinesterase inhibitor than the trans isomer (Table 4).

The selective toxicity of the thiono Phosdrin isomers can be understood on the basis of their activation and also on the comparative considerations in evaluating the toxicity of their oxygen analogs (see above).

It should be pointed out that the considerations outlined above, attempting to correlate toxicity with in vitro findings are open to criticism. The reason is that a) not all factors that could presumably be important in toxicity have been studied, and b) it is extremely difficult to evaluate the individual contribution of each factor in determining toxicity.

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