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INSECTICIDE METABOLISM

Metabolism of Malathion by Rat Tissue Preparations and Its Modification by EPN

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Homogenates of 11 rat tissues metabolized malathion at comparable rates and to similar metabolites. The main hydrolysis occurred at the carboxyester linkage. The hydrolysis at this linkage, the over-all hydrolysis, and the formation of malaoxon by various tissues in vitro were all inhibited by EPN in vivo. The synergism of EPN and malathion in vivo is therefore probably not attributable to an increased level of malaoxon in the body, but to a greater persistence of malathion and malaoxon in the tissues.

POTENTIATION" OR "SYNERGISM" as it is called in this paper, with organophosphate insecticides in mammals is of considerable interest, because of possible health hazards (*6*) and of the need for investigating the physiological background of the phenomenon (*3, 4, 10*).

Combinations of malathion and EPN deserve particular interest as after the initial report of Frawley *et al.* (*6*) it has frequently been confirmed that certain combinations of these two insecticides in the dog, rat, and mouse resulted in some remarkable synergistic effects (*5, 13*). It has been shown by Cook *et al.* (*3*) that malathion degradation by liver in vitro is inhibited by numerous organophosphates, and by Murphy and DuBois (*10*) that after EPN administration, the ability of the liver and serum to degrade malaoxon (the anticholinesterase which is a metabolite of malathion) is inhibited. Both groups, after studies on rats, concluded that their findings accounted for the observed synergistic effects.

The present study was initiated by the need for a broad approach to the metabolic background of the phenomenon, especially to locate the point of attack of EPN upon the metabolism of malathion. According to Cook and Yip (*4*), the principal liver metabolite of malathion in vitro is one of the half-esters—*O, O*-dimethyl *S*-(1-carboethoxy-2-carboxyethyl) phosphorodithioate. However, their Table I suggests that, in fact, more than one metabolite was produced; if this were so, it would reopen the problem of where EPN attacks.

This study examines the hypothesis that EPN inhibits degradation of malathion and/or malaoxon, and consequently raises the level of malaoxon in tissues. Such an effect in vivo would explain the potentiation of malathion by EPN.

Materials and Methods

Holtzman albino male rats, 160 to 180 grams, were used as sources of tissues which were pooled from at least two animals for every experiment. The animals were decapitated and the tissues immediately chilled after removal. The heparinized blood was separated into plasma and corpuscles (whole corpuscular fraction) by centrifugation.

The tissue preparations used were:

Natural form (plasma and corpuscles).

Homogenates, made with a Lourdes stainless steel homogenizer. Homogenates were used, because they were expected to yield primarily information on degradation. They were also the only type of preparation which could be used for all tissues.

Slices (thickness 0.2 mm.), cut with a Mickle tissue slicer, were used, because it was anticipated that they would yield information primarily on the balance between malathion oxidation and degradation (the procedure of slicing helps preserve cofactor-sensitive oxidative enzyme systems).

Acetone powders. One part of chilled tissue was homogenized in 20 parts of acetone at −50° C. and filtered, and the residue was rehomogenized twice in fresh, cold acetone portions. Acetone was removed by a Labline evaporator, and the powder was dried overnight under room conditions. Incubation suspensions were made up by homogenizing the acetone powders in isotonic solution. Acetone powders were used for comparison with the findings of Cook *et al.* (*3*) which were partially based upon this type of preparation.

Liver cell component fractions. Liver was homogenized in a Potter-Elvehjem type glass homogenizer and differentially centrifuged essentially according to Schneider (*14*); the preparation and incubation medium was isotonic sucrose solution (A) as specified below.

In experiments where only liver was used, not comparatively with other tissues, the liver was perfused before preparation with cold 0.9% sodium chloride solution in situ via the portal and hepatic veins in order to remove blood.

The incubation conditions were, unless stated otherwise: fresh tissue preparation (or the equivalent of fresh whole tissue as acetone powder or live cell component fractions) incubated at a concentration of 5% in isotonic solution A (0.018*M* calcium chloride in 0.25*M* sucrose) containing $6.8 \times 10^{-4}M$ P^{32} malathion for 30 minutes at 37.5° C. while shaken at 120 cycles per minute.

In experiments especially indicated below, a buffered isotonic solution, referred to as B, was used as incubation medium: 0.15*M* sodium chloride, 0.154*M*

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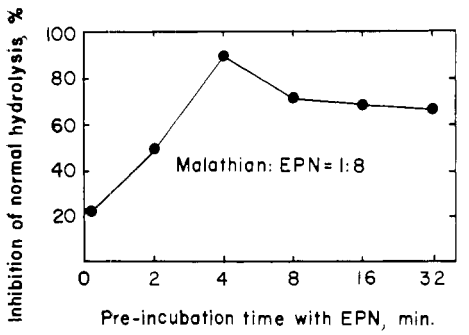

Figure 1. Effect of preincubation with EPN upon malathion hydrolysis

Malathion, 200 γ added after preincubation with 1600 γ EPN in isotonic solution A containing 5% liver homogenate

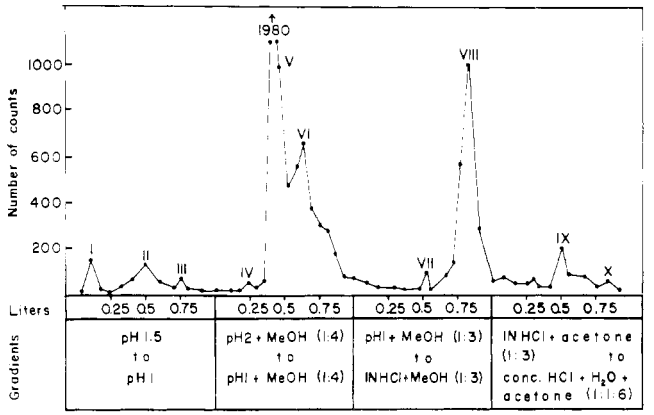

Figure 2. Ion exchange separation of water-soluble metabolites of P^{32} malathion

Liver acetone powder of untreated rats was incubated 30 minutes in isotonic solution A. Gradients indicated were applied in all ion exchange columns throughout the study

Peak No.	Metabolite
I	Phosphoric acid
II	Monomethyl phosphate
III	Dimethyl phosphate
IV	Unidentified
V	O,O-dimethyl S(1-carboethoxy-2-carboxy)ethyl phosphorodithioate or malathion monoacid
VI	O,O-dimethyl S(1,2-dicarboxy)ethyl phosphorodithioate or malathion diacid
VII	Unidentified
VIII	O,O-dimethyl phosphorothioate
IX	Unidentified
X	O,O-dimethyl phosphorodithioate

potassium chloride, 0.11*M* calcium chloride, 0.154*M* potassium acid phosphate, 0.154*M* magnesium sulfate heptahydrate, 0.1*M* phosphate buffer (pH 7.4), 2% Triton X-100, 0.36*M* glucose.

EPN (E.I. du Pont de Nemours and Co., Wilmington, Del.) was recrystallized *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothionate.

Synthesis of P^{32} Malathion. Phosphoric acid containing P^{32} (100 mc.) dissolved in hydrochloric acid was evaporated to dryness with dry air in a round-bottomed reaction flask, 0.25 gram of phosphorus pentasulfide was added, a condenser attached, and the flask was gassed with dry carbon dioxide. The contents were heated gently in the beginning, then vigorously for 10 additional minutes. Yellow crystals of phosphorus pentasulfide containing P^{32} formed on cooling (*2*). Five milliliters of toluene and a stirring bar were added to the reaction flask, the condenser was replaced, and 0.2 ml. of anhydrous methanol dissolved in 2 ml. of toluene was added dropwise. The contents were refluxed between 80° and 90° C. until all $P_2{}^{32}S_5$ had reacted.

The temperature was reduced to 50° to 60° C. and 0.425 ml. of diethyl maleate in 3 ml. of toluene were added. The mixture was refluxed for 4 hours at 85° C. with stirring. The residue was concentrated by evaporation, then taken up in chloroform and washed with an equal amount of 10% sodium carbonate. The compound was purified by chromatography on a Celite column with iso-octane–methanol (*1*) as eluent. The radioactive material was obtained in 70% yield and with an activity of 15,000 counts per minute per microgram.

Extraction Procedure. The protein of the incubation suspensions was precipitated and the pH was adjusted to 2.5 by saturated trichloroacetic acid solution. The suspension was partitioned against 30 ml. of chloroform, 3 grams of Celite were added, the suspension was filtered through a Büchner funnel, and the precipitate was washed alternately with 50 ml. each of water and chloroform. The precipitate was washed on another filtering flask with 20 ml. each of methanol and acetone, the washings were evaporated to dryness and taken up in the water and chloroform fraction, which was then centrifuged, and the water layer was removed. The chloroform was repartitioned against fresh water at pH 9 and centrifuged, and the water fractions were combined. An average of 5% of the total counts were not extractable from the protein-Celite filter residue.

The efficiency of the partitioning procedure was checked: (1) by repeated repartitions with fresh portions of water and chloroform, respectively: less than 2% of the total counts were found in the second partitioning step; (2) by partitioning a liver homogenate incubation suspension which had been inactivated (precipitated) prior to incubation: 0.4% of total counts were in the average found in water, and 99.8% in the malathion fraction—i.e., extracted by chloroform, and eluted by benzene from the aluminum oxide column (see below).

Column Chromatography. Water-Soluble Metabolites. The method described by Plapp and Casida (*12*) was employed with some slight modifications: An ion exchange resin (Dowex No. 1-X 8, 100 to 200 mesh, medium porosity, washed and stored in hydrochloric acid at pH 3) in a chromatography tube (21 mm. in diameter, 250 mm. long) was used with 3-pound pressure. The elution gradients are shown in Figure 1. Seven-milliliter samples were collected and counted with a Tracerlab 64 scaler or a Nuclear Chicago automatic unit.

The various water-soluble metabolites were tentatively identified by cochromatography with known compounds, whose position was determined by phosphorus assay. The possibility that one of the "unidentified" metabolites was malaoxon was examined, but it was found that this compound was not eluted off the column by the gradients shown in Figure 2 which were applied throughout the study.

Chloroform-Soluble Materials. Anionotropic aluminum oxide, activity grade 1, was used in column tubes 10 mm. in diameter and 170 mm. long. Two methods of adsorbent preconditioning have been employed: Aluminum oxide was thoroughly mixed with 2% (v./w.) water, slurried in hexane, and filled into the tube. The sample of chloroform-solubles, evaporated to dryness, and taken up in hexane, was added to the column. Two hundred milliliters each of hexane, benzene (eluting malathion), and chloroform (eluting malaoxon) were then run through successively one after another. As the aluminum oxide after such preconditioning retained between 20 and 55% of the radioactive material, another method based on the data of Malina *et al.* (*8*) was developed which resulted in 2 to 10% adsorption. Aluminum oxide was thoroughly mixed with 15% water, slurried in hexane, and filled into the tube. Unlabeled malathion (0.2 gram) in 1 ml. of hexane was added and 50 ml. of hexane were run through.

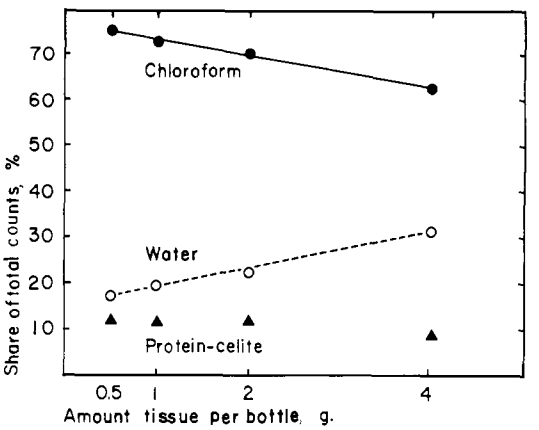

Figure 3. Hydrolytic activity as function of tissue concentration

Indicated amounts of liver homogenate per 20 ml. of suspension incubated for 30 minutes in isotonic solution A at 37.5° C.

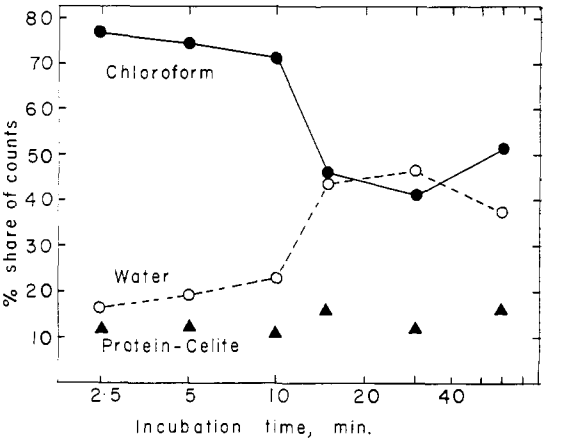

Figure 4. Hydrolytic activity as function of incubation time

Liver homogenates incubated as 5% of incubation suspension A for indicated times at 37.5° C.

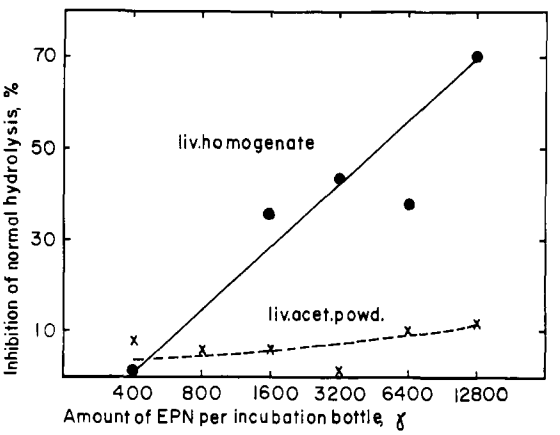

Figure 5. Influence of concentration of EPN upon degree of malathion hydrolysis in simultaneous incubation

Malathion, 400 γ per 20 ml. of liver homogenate suspension, 30 minutes of incubation.

Subsequently, the labeled sample taken up in hexane was added to the column and eluted with 50 ml. of hexane, 150 ml. of benzene, and 200 ml. of chloroform.

The ratio of malathion to malaoxon eluted off by both techniques was found to be equal.

The properties of the eluted malathion and malaoxon were identical with those reported by Gunther and Blinn (*7*), March *et al.* (*9*), and O'Brien (*11*) as checked by cholinesterase assays and infrared spectra.

Results

Favorable conditions for tissue concentration and incubation times were first established. Figure 3 shows that the extent of hydrolysis varied linearly with homogenate concentration: 2 grams of fresh weight tissue (or equivalents, see above) at 5% final concentration were used throughout subsequent experiments. Figure 4 shows that after 15 minutes of incubation, the hydrolysis has already leveled out at about 50% under the conditions stated. To ensure that despite varying conditions the incubation time was sufficiently long, and as respiratory experiments on slices showed regular oxygen consumption up to 50 minutes, 30 minutes were chosen as the standard incubation time.

Homogenates of various tissues were assayed. Table I shows the malathion hydrolyzing and oxidizing activities. The number and amounts of the water-soluble metabolites for some of them are listed in Table II, parts 1 to 3. All preparations listed formed water and chloroform-soluble metabolites of the same nature and to comparable extents. It was most surprising to find that homogenates exerted substantial oxidative properties. Contrary to what was anticipated from previous reports (*10*), liver was not one of the most hydrolytically and oxidatively active preparations. However, for comparison's sake with other reports, liver preparations were used as standard throughout the study. In confirmation of a previous study (*11*), the microsomes were the most effective fraction of liver in oxidizing malathion.

Effect of LD_{50} of EPN. For investigating the influence of EPN in vivo upon malathion metabolism in vitro, rats were injected with 40 mg. per kg. of EPN intraperitoneally and sacrificed after 5 hours of exposure (when symptoms were at their peak). This high dose was chosen to maximize the biochemical effects. As Table I shows, both hydrolysis and oxidation were inhibited to various degrees in all the preparations assayed. In every case, there was a net reduction in malaoxon production, rather than the anticipated increase.

The influence of various concentrations of EPN in the incubation medium upon malathion hydrolysis by liver homogenates and acetone powders was studied. Figure 5 shows that the relationships between concentration of EPN and inhibition of malathion hydrolysis were proportional, but with different factors for the two types of preparation. For one combination of EPN with malathion in vitro, the number and amounts of water-soluble metabolites are listed in part 4 of Table II. The formation of the monocarboxylic acid was severely inhibited, and phosphatase products, particularly dimethyl phosphate, increased correspondingly.

The influence of EPN in vitro and in vivo upon malathion metabolism in vitro was expected to vary with time of exposure, because of formation of metabolites of EPN whose effectiveness would possibly not be identical with that of EPN itself. Figure 5 shows that in vitro up to 4 minutes of preincubation with EPN before addition of malathion

resulted in increased inhibition of malathion hydrolysis, whereas longer preincubation times were less effective, probably as a result of the formation of less active EPN metabolites.

The results of the corresponding in vivo experiments are shown in Table III as far as the extent of hydrolysis and malaoxon formation are concerned, whereas Table II, part 5, shows the alterations in the nature and amounts of the water-soluble metabolites of malathion. As more pronounced effects with respect to the malaoxon levels were found with slices (instead of homogenates) incubated for 45 minutes, (instead of 30 minutes) in the buffered isotonic solution B (instead of A) these were selected for further study. The most significant metabolic shift caused by EPN was that from the normal of 35% carboxylic acid derivatives to 13% at 5 hours of exposure time to EPN and corresponding alterations in other metabolic pathways, indicating that it was mainly carboxyesterase which was inhibited by EPN. Formation of the monocarboxylic acid derivative, in particular, was severely inhibited.

There is good synchronization of the maximum peak of symptoms, minimal malathion hydrolyzing and oxidizing activity (Table III), and minimum in carboxylic acid derivatives (Table II, part 5). A peculiar stimulation of hydrolysis activity was repeatedly found at 125 hours, perhaps reflecting stimulated synthesis of esterases.

Effect of Low Dose of EPN. The unexpected finding that EPN actually depressed the level of malaoxon in all tissue preparations studied gave rise to the suspicion that such an effect was due to the use of a relatively high dose of EPN. Rats were therefore treated with $^1/_{10}$ of the LD_{50} of EPN—i.e., 4 mg. per kg.—and a comparative experiment was set up, the results of which are presented in Table IV. There was a depression of the extent of hydrolysis, the percentage of chloroform-solubles as malaoxon, and the absolute level of malaoxon. These effects were usually less after 4 mg. per kg. than after 40 mg. per kg. of EPN.

As these results obtained with liver preparations were somewhat unexpected, analogous assays were conducted with homogenates of nonhepatic tissues, under the same conditions as those under

Table I. Effect of LD_{50} of EPN in Vivo upon Subsequent Hydrolysis and Oxidation by Rat Tissue Homogenates Compared with Those of Untreated Rats

Preparation	Hydrolysis: Normal, % of total counts as water-solubles	Hydrolysis: EPN treated, % inhibition of normal, 5 hr. after 40 mg./kg. EPN	Malaoxon: Normal, % of total counts as malaoxon	Malaoxon: EPN treated, % inhibition of normal, 5 hr. after 40 mg./kg.EPN
Liver acetone powder	77	21	4	64
Kidney homogenate	66	85	4	50
Heart homogenate	61	23	...	...
Blood plasma	60	52	9	83
Ileum homogenate	59	0	...	...
Spleen homogenate	58	38	...	...
Lung homogenate	57	86	...	...
Testis homogenate	56	41	10	20
Muscles (diaphragm) homogenate	55	60	...	...
Blood corpuscles	53	62	...	...
Liver homogenate	52	14	7	86
Brain homogenate	41	0	8	83
Liver				
Nuclear fraction	71	...	2	...
Mitrochondrial fraction	69	...	4	...
Supernatant	52	...	1	...
Microsomal fraction	45	...	11	...

Preparations incubated in isotonic solution A for 30 minutes at 37.5° C.

Table II. Malathion Hydrolysis Products Formed by Rat Tissue Preparations in Vitro

Part	Preparation		Phosphoric Acid Derivatives[a] I	II	III	VIII	X	Total[b]	Carboxylic Acid Derivatives[a] V	VI	Total[b]	Unidentified Metabolites[a] IV	VII	IX	Total[b]
1	Liver homogenate		<1	1	0	0	3	4	30	53	83	<1	<1	11	11
	Lung homogenate		<1	<1	1	9	<1	10	33	55	88	0	1	<1	1
	Kidney homogenate		<1	<1	0	9	<1	9	39	50	89	0	1	<1	1
	Blood plasma		<1	<1	0	7	<1	7	50	42	92	0	0	0	0
	Brain homogenate		1	1	3	18	1	24	18	52	70	1	1	4	6
2	Liver slices		2	0	0	15	20	37	19	41	60	1	0	2	3
3	Liver acetone powder		2	3	0	<1	<1	5	32	38	70	0	19	5	24
4	Liver homogenate + 200 γ malathion + 2000 γ EPN		1	1	1	24	1	28	6	54	60	3	1	8	12
5	Liver homogenates from rats after 40 mg./kg. EPN intraperitoneally sacrificed after indicated times	Untreated	<1	1	0	0	3	4	30	53	83	<1	<1	11	11
		0.2 hr.	1	3	2	41	11	58	5	30	35	5	1	1	7
		1	2	10	6	28	3	49	2	13	15	2	7	27	36
		5	4	3	7	29	2	45	1	12	13	1	10	31	42
		25	<1	2	9	30	8	49	3	11	14	4	8	24	36
		125	2	<1	2	2	3	9	4	36	40	1	27	23	51

Results are as fraction of total water-solubles in per cent.

[a] Roman numerals indicate metabolites listed in Figure 5.

[b] Numbers for total amounts within a group are approximations, as metabolite levels below 1% were not determined precisely.

Table III. Dependency of Malathion Hydrolysis and Oxidation by Liver Slices in Vitro upon Time of Exposure to EPN in Vivo

	Normal	0.2 Hr.	1 Hr.	5 Hr.	25 Hr.	125 Hr.
Water-soluble metabolites	65.7	61.0	51.0	31.2	42.8	92.0
Chloroform-soluble metabolites	31.2	36.0	42.3	60.4	47.8	5.2
Retained in protein-Celite	3.1	3.0	6.7	8.4	9.4	2.8
Malathion	17.4	19.8	38.5	60.2	45.4	4.1
Malaoxon	13.8	16.2	3.8	0.3	2.4	1.1

40 mg./kg. EPN injected intraperitoneally. Liver slices were incubated with malathion in the buffered isotonic solution B for 45 minutes. Results are per cent of total counts.

which liver had been tested—45 minutes of incubation in buffered isotonic solution B. Table V gives information on the extent of hydrolysis and malaoxon levels normally and after EPN at $^1/_{10}$ LD_{50}; as with liver, hydrolytic and oxidative activity were both depressed in these tissue preparations. The depression of both the oxidation and hydrolysis was particularly marked in brain.

Discussion

The study has shown that a variety of preparations of hepatic and nonhepatic tissues are capable of malathion hydrolysis and oxidation in vitro. Although there are quantitative rather than qualitative differences between the same type of preparation of various tissues and between different types of preparation of the same origin, there is a surprisingly narrow range of deviation. Ten water-soluble degradation products have been found, of which the monoacid and diacid derivatives are the most abundant in all tissues examined. The fact that so many tissue preparations were capable of breaking down malathion to comparable extents may account for the rapidity of malathion detoxification in vivo (9).

All tissue preparations from rats treated with the LD_{50} of EPN showed significant depression of malathion hydrolyzing and oxidizing activity, the maxima of which coincided with the maximum of intensity of externally visible symptoms. Treatment with EPN at one tenth of the LD_{50} affected the hydrolyzing and oxidizing activities to a significant but smaller extent and did not result in externally visible symptoms. There are significant differences in the extents of hydrolysis, malaoxon levels, and water-soluble metabolites depending merely on the type of preparation or treatment; whereas with liver slices the phosphoric acid derivatives amount to about 35% of the total radioactivity, they constitute only about 5% in homogenates and acetone powders (Table II, parts 1, 2, and 3). The values for the carboxylic acid derivatives range between 60 and 80% and for unidentified metabolites from 3 to about 25%. A more conspicuous difference is represented in Figure 4, which shows that EPN has a very small effect upon metabolism by liver acetone powders as compared to homogenates. These extracts are probably unable to oxidize EPN to a potent inhibitor. Alternatively, acetone extraction may inhibit or remove a system which degrades malathion and which is sensitive to EPN.

The metabolic shift in water-soluble metabolites induced by EPN in vitro and in vivo (from abudant formation of carboxylic acids to more phosphoric acid derivatives, parts 4 and 5, Table II) indicates that carboxyesterase(s) is/are primarily inhibited by EPN.

The malaoxon levels determined are resultants of two oppositely directed processes: the oxidative formation from malathion and the hydrolytic decomposition to water-soluble products. In the case of homogenates and acetone powders, the oxidizing system was probably working under suboptimal conditions, because various cofactors are known to be of importance in such systems (*11*). But with liver slices a clear reduction of malaoxon formation was also noted, and an inhibition by EPN of the oxidation of malathion must be inferred. The explanation for this could be a competition of both malathion and EPN for the same oxidizing system(s).

The tissues of rats treated in vivo with EPN thus show a reduced capacity to hydrolyze and oxidize malathion in vitro. This is true for the LD_{50} or $^1/_{10}$ LD_{50} of EPN, for various exposure times, for the 11 tissues studied and for different types of preparation. There is, therefore, no evidence from these in vitro studies to support the hypothesis that synergism between EPN and malathion observed in vivo is due to a generally increased level of malaoxon resulting from inhibition of malathion and malaoxon hydrolysis. The data are compatible with an alternative hypothesis—namely, that EPN by virtue of its inhibition of hydrolysis and oxidation causes a greater persistence of malathion in the body tissues. The synergistic effect itself, however, must be due to an increased level or persistence of malaoxon at the target site; presumably the persistence of malathion in the various body tissues maintains the malaoxon level at the target for a much greater time, although that level may be somewhat lower than in the absence of EPN. Vandekar and Heath (*15*) were able to produce enhanced symptoms in rats and a much more irreversibly inhibited brain cholinesterase by artificially maintaining persistent body levels of another dimethyl phosphorothionate (*O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothionate). This procedure may be analogous to the effect of EPN.

Table IV. Effects of Two Dosages of EPN in Vivo at Two Exposure Times upon Malathion Hydrolysis and Oxidation by Liver Slices and Homogenates in Vitro

	Hydrolysis Products		Malaoxon	
	Liver slices	Liver homogenates	Liver slices	Liver homogenates
Normal	65.6 ± 1.3	84.4 ± 3.3	14.1 ± 1.1	13.3 ± 0.9
4 mg./kg. EPN				
1-hr. exposure	90.8 ± 0.1	86.2 ± 2.1	1.5 ± 0.1	1.9 ± 0.02
5-hr. exposure	88.5 ± 2.8	81.4 ± 8.4	2.6 ± 0.3	4.8 ± 1.3
40 mg./kg. EPN				
1-hr. exposure	55.6 ± 7.5	60.4 ± 12.9	6.6 ± 1.4	9.1 ± 1.4
5-hr. exposure	33.2 ± 2.5	29.0 ± 7.1	6.4 ± 2.1	0.82 ± 0.01

All preparations incubated for 45 minutes in buffered isotonic solution B. Results are per cent of total counts, ± standard error. At least 4 animals used for each determination.

Acknowledgment

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Table V. Effect of 1/10 LD_{50} of EPN in Vivo upon Subsequent Hydrolysis and Oxidation of Malathion in Vitro by Rat Tissue Homogenates

	Hydrolysis			Malaoxon		
	% of Total Counts as Water-Solubles			% of Total Counts as Malaoxon		
	Normal	4 Mg./Kg. EPN		Normal	4 Mg./Kg. EPN	
		Exposure Time			Exposure Time	
		1 hr.	5 hr.		1 hr.	5 hr.
Plasma	90.2 ± 5.3	50.6 ± 2.3	85.6 ± 3.1	4.4 ± 0.1	3.9 ± 0.3	3.0 ± 0.2
Kidney homogenate	85.2 ± 1.2	69.0 ± 11.1	76.4 ± 3.7	6.1 ± 0.9	3.2 ± 0.3	5.8 ± 0.3
Brain homogenate	85.0 ± 7.3	14.2 ± 1.2	76.5 ± 6.4	4.7 ± 1.0	0.8 ± 0.0	0.9 ± 0.1
Liver homogenate	84.4 ± 3.3	86.2 ± 2.1	81.4 ± 8.4	6.1 ± 1.3	3.2 ± 0.2	6.5 ± 0.2
Testis homogenate	83.8 ± 2.8	72.5 ± 4.6	86.3 ± 2.3	8.3 ± 1.3	7.9 ± 0.7	7.1 ± 1.1

Tissue homogenates were incubated in buffered isotonic solution B for 45 minutes. Results are per cent of total counts.

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INSECTICIDE METABOLISM

Prechromatographic Purification of Insecticides from Insect Tissue Extracts

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Paper chromatograms of acetone extracts of insecticides and their metabolites from insect tissues often are smeared and unrecognizable. When a crude extract is spotted on filter paper and eluted with acetonitrile, the solvent selectively extracts insecticides, metabolites, and 20 to 30% of the insect lipide material, but not the interfering lipides. The purified extracts give clear chromatograms, on which insecticides and metabolites can be identified. This rapid and simple purification is very useful in studies of insecticide metabolism.

PAPER CHROMATOGRAPHY is used in the study of insecticide metabolism in plants, insects, and higher animals. In the course of studies on the metabolism of DDT, Systox, Thimet, and Sevin in houseflies, mosquitoes, German roaches, and milkweed bugs, it became evident that acetone extracts of insect tissues contain large amounts of lipoid material which causes smearing of paper chromatograms. This results in poorly defined spots, incomplete separation of spots, and shifting of R_f values. Therefore a method had to be devised for removing interfering materials from the extract before running a paper chromatogram. The acetonitrile method described in this paper has been successful with all the insecticides and insects so far studied in this laboratory, but only a few typical examples are presented here.

Procedure

Insects amounting to several hundred milligrams wet weight are homogenized in 5 ml. of acetone. A pinch of anhydrous sodium sulfate is added to remove water present in the homogenate, which is then centrifuged at 2000 r.p.m. for 20 minutes. The supernatant liquid is drawn off and the residue is re-extracted once or twice with 5 ml. of acetone. The combined volume (about 15 ml.) of acetone extract is evaporated in a stream of nitrogen gas to a final volume of 30 to 50 μl. and is transferred by self-filling capillary tubes onto the center of a strip of Whatman No. 4 chromatographic filter paper, 2.5 inches long and 1 inch wide, but at one end leading to a tapering point. Disposable glass capillaries are more convenient than micropipets, give faster transfer, and require no cleaning. One microliter of a 0.5% solution of *N,N'*-dimethyl-*p*-1-naphthylazoaniline (NDN) in benzene is spotted over the extract spot. In tests using carbon-14–DDT, carbon-14–Systox, and Thimet (detected by the sensitive color reaction with 2,6-dibromoquinonechloroimide), in extracts of houseflies and German roaches, it was found that when the NDN was completely extracted from the spot, no insecticide was detectable in the spot, but all the insecticide moved with the dye. The dye is a useful visual indicator of complete extraction. Both dye and insecticides move out of the spot gradually as very diffuse bands, by a slow elution process, probably analogous to a Soxhlet extraction.

Extraction is effected either by an upward-washing or a downward-washing method. The former is more convenient, but requires that the entire sample be spotted on one chromatographic strip. The latter allows the use of several aliquots of the sample on several strips if desired.

Upward Washing. The broad end of the spotted strip is dipped into a 50-ml. beaker containing 5 to 10 ml. of acetonitrile. The ascent of this solvent by capillarity carries insecticide, reference dye (NDN), and some lipoid material to the pointed tip of the strip. The bulk of the lipoid material stays in the original spot. The strip is held upright by clamping between two glass microscope slides (3 × 1 inch). Solvent is transferred from the tip by contact with a paper chromatographic strip to form a spot. This is dried and more solvent transferred to it repeatedly until transfer is complete—as indicated by complete removal of the dye from the tip (*5*).

Downward Washing. The spotted strip is hung in a closed tube in a cup containing acetonitrile, with the tip pointing down and touching a glass rod standing in a receiving cup (Figure 1). After 0.5 hour the solvent is collected from the receiving cup, concentrated to a small volume, and spotted on a paper chromatographic strip.

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