Metabolism of Some Organophosphorus Insecticides

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Dicrotophos, monocrotophos, phosphamidon, and dimethoate are oxidatively dealkylated *in vivo* to give products which are progressively more toxic to mice and houseflies. With sesamex, the dialkylamides, monoalkylamides, and unsubstituted amides are of similar toxicity to houseflies. CIBA's C-2307, in which the $-N(CH_3)_2$ of dicrotophos is replaced by $-N(CH_3)(OCH_3)$, is also metabolized to give the unsubstituted amide analog, but the sequence of metabolic reactions is quite different from dicrotophos. Phosphamidon is also dechlorinated to give products where the vinyl chlorine is replaced by an hydroxyl group. The *in vitro* degradation of malathion, dimethoate, and diazinon is also discussed. Malathion is hydrolyzed by a liver carboxylesterase, dimethoate by a microsomal amidase, and diazinon by a microsomal mixed-function oxidase system, all from mammalian liver. Properties and substrate specificity of the enzymes are described. The metabolic fate of the three insecticides in mammals and insects is related to their selectivity.

The metabolism of organophosphorus insecticides can be conveniently divided into reactions which result in hydrolysis of the parent molecule and subsequently less toxic products, and those reactions, primarily oxidative, which result in neutral products which retain at least a degree of toxicity. Reactions of the first type are the predominant ones in a quantitative sense, and have been extensively investigated (Heath, 1961; O'Brien, 1960, 1967). On the other hand, reactions leading to neutral metabolites are less well understood, although from a toxicological standpoint they are more important. The oxidative conversion of phosphorothionates to phosphates is a notable exception to this generalization. Many workers have studied this reaction in detail, and it seems to be well understood (Heath, 1961; O'Brien, 1960, 1967). However, there is the possibility in many compounds of other kinds of oxidation which are not so well understood and are important.

In general, the initial studies on the metabolism of organophosphorus insecticides have been conducted in whole plants and animals *in vivo* (Heath, 1961; O'Brien, 1960, 1967). Normally, animals are treated with a labeled insecticide, and the balance of excreted radioactivity in the urine and feces is determined, after which the animal is sacrificed and the residual radioactivity is determined in the tissues. Identification of metabolites excreted in the urine, feces, and sometimes milk is a major part of these studies. After balance studies and identification of new metabolites (Dauterman *et al.*, 1959; March *et al.*, 1956; Plapp and Casida, 1958), then *in vitro* studies have been undertaken. In a few cases, *in vitro* studies have been initiated to attempt to explain the cause of selectivity of certain organophosphorus compounds.

In vitro studies have the advantage of considering an isolated portion of the metabolism of a compound. By isolating the enzyme or system responsible for the biochemical alteration of the parent compound, one can determine optimum conditions, structure-activity relationships, and obtain a better understanding of the detoxication system, with the hope of utilizing these systems to the advantage of man and animal in the design of new insecticides.

In the first part of this paper we will consider the *in vivo* metabolism of a number of substituted amide organophosphorus insecticides, and in the second part will consider three *in vitro* systems that have been used for the investigation of organophosphorus insecticide metabolism.

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METABOLISM OF SUBSTITUTED AMIDE ORGANOPHOSPHORUS INSECTICIDES *IN VIVO*

Phosphoramidates. One of the earliest substituted amide organophosphate compounds was schradan (octamethylpyrophosphoramide). It was noted in the early work on this compound that there is a metabolite that is many times more toxic than the parent compound. Hartley (1951) proposed that this metabolite was the *N*-oxide of schradan, which was subsequently rearranged to give an *N*-hydroxymethyl derivative. Casida and coworkers (Casida *et al.*, 1954, Tsuyuki *et al.*, 1955) presented evidence supporting the *N*-oxide structure as being the active anticholinesterase. They also presented evidence for the $N\text{-}O\text{-}CH_3$ structure for a rearrangement product of the *N*-oxide (Tsuyuki *et al.*, 1955) although Spencer *et al.* (1957) synthesized this compound and found it to have different properties than the metabolite. Later work, however, has indicated that most probably the correct structure for this metabolite is the *N*-hydroxymethyl schradan (Heath *et al.*, 1955; Spencer *et al.*, 1957). Heath (1961) summarizes the evidence for and against the various proposed structures for the schradan metabolite. Dimefox [bis(dimethylamido) fluorophosphine oxide] is metabolized to give similar products as schradan (Arthur and Casida, 1958; Casida *et al.*, 1953; Fenwick, 1958), although it has not been so thoroughly investigated.

Since this early work, a number of other organophosphorus insecticides have been introduced that possess the substituted amide moiety, although they are not phosphoramidates. Among these are dimethoate [*O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorodithioate], phosphamidon (2-chloro-*N,N*-diethyl-3-hydroxycrotonamide, dimethyl phosphate), dicrotophos (3-hydroxy-*N,N*-dimethyl-*cis*-crotonamide, dimethyl phosphate), monocrotophos (3-hydroxy-*N*-methyl-*cis*-crotonamide, dimethyl phosphate), which is the monomethylamide analog of dicrotophos, and CIBA's C-2307 (3-hydroxy-*N*-methyl-*N*-methoxycrotonamide, dimethyl phosphate).

Metabolism of Dicrotophos and Monocrotophos. The first complete elucidation of the oxidative dealkylation of a disubstituted amide insecticide was reported for dicrotophos by Menzer and Casida (1965). Identification of metabolites was facilitated in this work by the use of mixed ^{32}P- and *N*-methyl-^{14}C-labeled dicrotophos and monocrotophos, since the loss of labeled methyl groups could then be followed and

Table I. Biological Activity of Dicrotophos and Its Analogs[a]

cis-$(CH_3O)_2P(O)OC(CH_3)$=CHC(O)R	LD_{50}, mg/kg		
	Female Mouse, i-p	Housefly, topical[b] −Sesamex	+Sesamex
R = $N(CH_3)_2$ (dicrotophos)	14	38	1.0
R = $N(CH_2OH)CH_3$[c]	18	14	1.2
R = $NHCH_3$ (monocrotophos)	8	6.4	0.8
R = $NHCH_2OH$[c]	12	30	3.4
R = NH_2	3	1.0	0.9

[a] Menzer and Casida (1965). [b] Fly LD_{50} values for organophosphates determined with and without simultaneous treatment with 10 μg sesamex per fly. Slopes for log dose-probit mortality lines were similar for all compounds with and without synergist. [c] Impure synthetic products.

the ratio of ^{32}P to ^{14}C in metabolites could be used to indicate the state of demethylation. This has been a very useful technique in later work on other compounds also. Dicrotophos was shown to undergo oxidation to form an *N*-hydroxymethyl derivative. The hydroxymethyl group was subsequently lost to give monocrotophos, the monomethyl derivative, which also underwent oxidation to its *N*-hydroxymethyl derivative. Loss of this group then gave the unsubstituted amide analog of dicrotophos and monocrotophos. The importance of these metabolites is illustrated in Table I. As the methyl groups are removed, the resulting metabolites are more toxic to both mice and houseflies (*Musca domestica* L.). When houseflies are pretreated with sesamex [2-(2-ethoxyethoxy)ethyl 3,4-(methylenedioxy) phenyl acetal of acetaldehyde] before treatment with dicrotophos and its metabolites, the toxicity of all compounds is of the same order. The dimethylamide is synergized about 40 times, the monomethylamide, about eight times, and the unsubstituted amide is not synergized at all.

Metabolism of Phosphamidon. Phosphamidon is very similar to dicrotophos in structure, except that it is a diethylamide instead of a dimethylamide. The vinyl hydrogen of dicrotophos is also replaced by a vinyl chlorine in phosphamidon. The metabolism of phosphamidon was investigated originally by Anliker and coworkers (1961), but the only neutral organophosphorus metabolite detected was the monodeethylated product. In more recent work reported by Bull *et al.* (1967), Clemons and Menzer (1968), and Lucier and Menzer (1970b), the metabolism of phosphamidon has been shown to be more complicated. Three differently labeled samples of phosphamidon were used in the recent work: phosphamidon-^{32}P, phosphamidon-α-diethylamide-^{14}C, and phosphamidon-vinyl-carbonyl-^{14}C [$(CH_3O)_2P(O)O^{14}C(CH_3)$=$C(Cl)^{14}C(O)N(C_2H_5)_2$]. At least seven phosphorus-containing metabolites were detected in rat urine, and in goat milk and urine after administration of mixed phosphamidon-^{32}P and -*N*-α-diethylamide-^{14}C. Separation was achieved on Celite partitioning columns using hexane-chloroform mixtures for elution (Figure 1). In the early stages of the work, only the mono-*N*-ethyl metabolite, metabolite II, and the unsubstituted amide metabolite, metabolite V, could be unequivocably characterized (Clemons and Menzer, 1968). It proved to be very difficult to synthesize the hydroxyethyl intermediate metabolites of phosphamidon, although there were strong indications that they were present. Metabolites VI and VII were both found to form labeled dimedone derivatives in stoichiometric quantities after hydrolysis, indicating the presence of an *N*-hydroxyethyl group on each metabolite, which was liberated as acetaldehyde-^{14}C upon treatment with acid. This fact, along with the ratio of ^{32}P to ^{14}C in the two metabolites, strongly suggests that VI is the *N*-ethyl-*N*-hydroxyethyl and VII is the mono-*N*-hydroxyethyl derivative of phosphamidon.

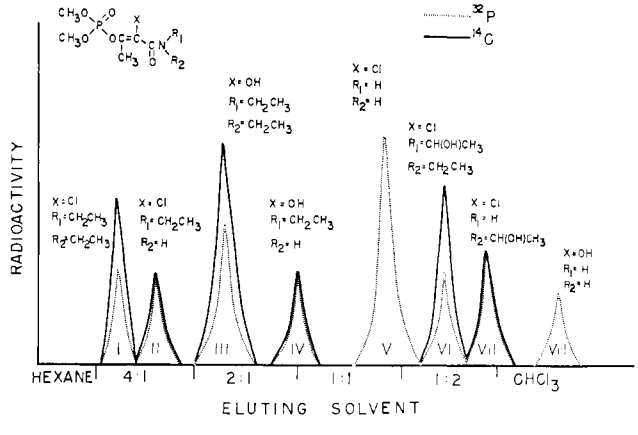


Figure 1. Representation of the separation of phosphamidon metabolites achieved on a Celite column based on partitioning between water and hexane-chloroform mixtures

This type of separation results from chromatographing the organoextractable fraction of animal extracts after treatment with mixed phosphamidon-^{32}P and phosphamidon-*N*-α-diethylamide-^{14}C

If one assumed that, of the seven metabolites, two were hydroxyethyl intermediates and the des-*N*-ethyl and unsubstituted amide metabolites accounted for two more, there still remained three metabolites unidentified which did not seem to fit into the oxidative *N*-dealkylation scheme but which were neutral phosphorus esters. One of these was present in relatively large amounts (metabolite III). Metabolites III, IV, and VIII were reacted with acetic anhydride, and less polar compounds resulted in each case. The three metabolites were also treated with concentrated hydrochloric acid. Metabolite III yielded phosphamidon, metabolite IV yielded desethyl phosphamidon, and metabolite VIII yielded the unsubstituted amide based on their chromatographic positions and rates of hydrolysis in 0.05*N* sodium hydroxide. These facts indicate that the vinyl chlorine had been replaced by an hydroxyl group, which could in turn be rechlorinated to give the corresponding phosphamidon analog. Spectral evidence and direct chemical evidence for these structures must still be obtained. Attempts to synthesize the vinyl hydroxyl analogs have been unsuccessful. The metabolic route of phosphamidon leading to neutral metabolites in these systems is illustrated in Figure 2.

Because of the difficulty in synthesizing the vinyl hydroxyl analogs and the *N*-hydroxyethyl analogs, biological data have not been obtained on all of the new metabolites. However, the earlier work on phosphamidon showed that the desethyl metabolite was approximately as toxic to rats as phosphamidon itself (Anliker *et al.*, 1961). Clemons and Menzer (1968) showed that phosphamidon and desethyl phosphamidon were approximately equitoxic by intraperitoneal injection to white mice (LD_{50} = 6.0 mg per kg), but that the unsubstituted amide had an LD_{50} of 2.5 mg per kg.

Dimethoate Metabolism. Many studies on the metabolism of dimethoate have appeared in the literature, but all deal primarily with the hydrolysis products (Allesandrini, 1962; Dauterman *et al.*, 1959, 1960; dePietri-Tonelli *et al.*, 1961; Hacskaylo and Bull, 1963; Rowlands, 1966; Sampaolo, 1961, 1962; Santi and Giacomelli, 1962). However, Sanderson and Edson (1964) reported the presence of a number of unidentified, organoextractable metabolites of dimethoate in

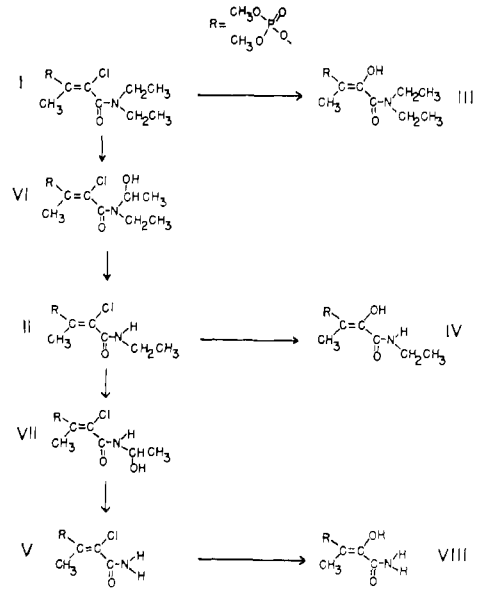


Figure 2. Proposed metabolic route of phosphamidon resulting in neutral metabolites in rats

the urine after the treatment of rats. They speculated that these metabolites might be the products of *N*-demethylation of dimethoate, although no definitive characterization studies were conducted. Lucier and Menzer (1968) reported on the fate of dimethoate in bean plants using several different modes of application of the chemical. Dimethoate-carbonyl-^{14}C and -^{32}P were used and three previously unreported metabolites were detected by using a combination of column and thin-layer chromatographic techniques. One of the metabolites was the des-*N*-methyl derivative of dimethoate, while the other two were speculated to be the *N*-hydroxymethyl derivatives of dimethoate and dimethoate oxygen analog. Oxidation of dimethoate took place to a lesser degree than hydrolysis, although it seemed more important in the foliar-treated plants than in the other modes of application: stem-injection, root-uptake, and excised leaf.

In an attempt to characterize the oxidative metabolites of dimethoate, the compound was prepared with an *N*-methyl-^{14}C label (Lucier and Menzer, 1970a). This was used in combination with dimethoate-^{32}P and dimethoate-carbonyl-^{14}C and administered to rats and bean plants. Four metabolite peaks in addition to a peak for dimethoate itself were observed after Celite column chromatography of chloroform extracts of rat urine (Figure 3). Peak II cochromatographed with des-*N*-methyl dimethoate and did not contain the *N*-methyl-^{14}C-label. Peak IV likewise cochromatographed with des-*N*-methyl oxygen analog and did not contain the *N*-methyl-^{14}C label. Peak V cochromatographed with *N*-hydroxymethyl oxygen analog, and contained both the carbonyl and *N*-methyl-^{14}C labels. It also formed a labeled dicoumarol derivative after hydrolysis, indicating the presence of an *N*-hydroxymethyl group. Peak III cochromatographed with both dimethoate oxygen analog and *N*-hydroxymethyl dimethoate. Analysis of the peak by hydrolysis and formation of a dicoumarol derivative revealed that part of the radioactivity in the peak was present as an *N*-hydroxymethyl group. On the basis of this information, peak III must contain both of these metabolites. No column or thin-layer chromatographic system that has been tried has been effective in separating these two compounds. From these data, therefore, the complete sequence of oxidative metabolism of dimethoate

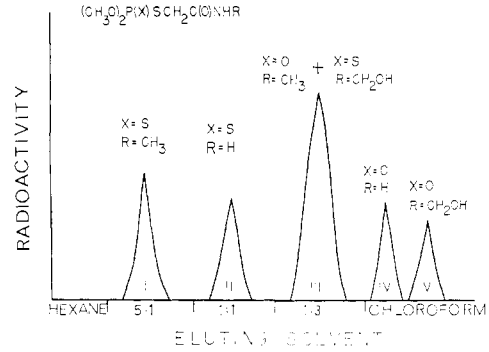


Figure 3. Representation of the separation of dimethoate metabolites achieved on a Celite column based on partitioning between water and hexane-chloroform mixtures

This type of separation results from chromatographing the organoextractable fraction of plant or animal extracts after treatment with dimethoate-^{32}P

has been shown to occur in the systems investigated (Figure 4). However, no dimethoate organoextractable metabolites were detected in rat urine after the first 24 hr following oral administration. At all sampling times most of the radioactivity was excreted as hydrolysis products. This points out the instability and transitory nature of these metabolites.

The toxicity of the dimethoate metabolites illustrates the importance of these studies (Table II). As expected, the oxygen analogs are the more toxic compounds to both mice and houseflies. The des-*N*-methyl oxygen analog is significantly more toxic than the oxygen analog itself to houseflies, although this is not true for mice. Sesamex pretreatment of houseflies makes the oxygen analog and the des-*N*-methyl oxygen analog approximately equitoxic to houseflies, in the same way that the dicrotophos metabolites were all rendered approximately equitoxic by pretreatment with sesamex.

Metabolism of C-2307. A newly developed compound, C-2307, of the Ciba Agrochemical Co., is very similar to dicrotophos. It differs only in having a methoxy group substituted for one of the amide methyls [$(CH_3O)_2P(O)OC(CH_3)$=$CHC(O)N(CH_3)OCH_3$]. Preliminary information on the metabolism of this compound has been obtained using only the ^{32}P-labeled material (Bosik and Menzer, 1970). Synthesis of the ^{14}C-labeled compound is under way. The early indications are that the *N*-methoxy group is not the initial site for metabolism of the molecule, since none of the mono-*N*-methyl metabolite is found in rat urine after treatment with C-2307. However, the unsubstituted amide is found, along with an unknown metabolite in which the amide moiety may be modified to —NH-OCH_3 or, less likely, —$N(CH_3)OH$. The identity of this unknown probably holds the key to the exact dealkylation route followed by C-2307.

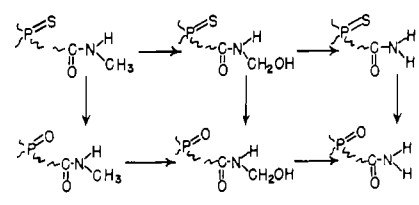


Figure 4. Proposed oxidative metabolic route of dimethoate in rats and bean plants

Only the relevant parts of the molecule are shown. The entire structure is $\underline{(CH_3O)_2}\sim P(S)\sim\underline{SCH_2}\sim C(O)\text{-}NHCH_3$ (Omitted portions underlined)

Table II. Biological Activity of Dimethoate and Its Metabolites[a]

$(CH_3O)_2P(X)SCH_2C(O)NHR$	LD$_{50}$, mg/kg[b] Mouse, Male, i-p	Housefly −Sesamex	Housefly +Sesamex
X = S R = CH_3	151(137–166)	0.83(0.72–0.95)	0.71(0.59–0.86)
X = S R = H	190(168–215)[c]	0.69(0.54–0.88)	0.84(0.54–1.28)
X = O R = CH_3	13(11–16)	0.21(0.17–0.25)	0.05(0.03–0.07)
X = O R = H	10(8–13)	0.09(0.07–0.12)[c]	0.06(0.04–0.08)

[a] Lucier and Menzer (1970a). [b] Numbers in parentheses are 95% confidence limits calculated by method of Litchfield and Wilcoxon (1949). [c] Value is significantly different from value immediately above at 5% level, values without letter designation are not significantly different from value immediately above.

Table III. Biological Activity of C-2307 Compared with Dicrotophos[a]

Insects

Insecticide	LD$_{50}$, mg per kg[b] Housefly, topical −Sesamex	Housefly, topical +Sesamex	German cockroach, topical −Sesamex	German cockroach, topical +Sesamex
C-2307	2.2(2.1–2.4)	0.53(0.50–0.55)	10(8.0–12.5)	1.8(1.6–2.0)
Dicrotophos	34(33.0–35.1)	0.88(0.81–0.95)	85(74.5–102.7)	5.0(4.0–6.3)

Mammals

Insecticide	LD$_{50}$, mg per kg[b] Female Mouse, Intraperitoneal Control	Female Mouse, Intraperitoneal With Phenobarbital
C-2307	1.3(1.15–1.45)	1.7(1.63–1.78)
Dicrotophos	11.2(10.3–12.2)	21.6(18.5–25.3)

[a] Bosik and Menzer (1970). [b] Numbers in parentheses are 95% confidence limits calculated by method of Litchfield and Wilcoxon (1949).

The insertion of the oxygen to give the —N-O-CH_3 compound apparently inhibits the oxidative demethylation of the amide group and acts as an "internal synergist." The biological activity of C-2307 is compared with dicrotophos in Table III. The toxicity of C-2307, with only this small difference in the molecule from dicrotophos, is remarkably greater to insects than dicrotophos. Furthermore, the degree of synergism when houseflies are treated with sesamex is not as great as that observed with dicrotophos, only about four times with C-2307 compared with about 38 times for dicrotophos, and six times with C-2307 in German cockroaches [*Blatella germanica* (L.)] compared with 17 times for dicrotophos. Pretreatment of mice for 3 days with 75 mg per kg of sodium phenobarbital per day also does not decrease the toxicity as much as it does with dicrotophos (Menzer and Best, 1968). Further work in progress on this compound should allow the development of some interesting insights into the sequence of oxidative dealkylation reactions.

Although the oxidative dealkylation reactions described here are relatively minor from a quantitative standpoint, they are of considerable importance because of the toxicity of the metabolites to both mammals and insects. We may also be able to develop some understanding of the mode of action of methylene dioxyphenyl synergism by studying the relationships that have been pointed out to exist with these compounds and their metabolites *vis-a-vis* their synergism. In addition, an understanding of the metabolism of C-2307 should enable us to understand the mechanism of oxidative dealkylation of other similar compounds, especially among the substituted phenylurea herbicides (Geissbühler, 1969), as well as elucidating the actual mechanism of oxidative dealkylation.

ORGANOPHOSPHORUS INSECTICIDE METABOLISM *IN VITRO*

Malathion-Carboxylesterase System. Malathion is hydrolyzed by an enzyme found in rat liver. The acid released on hydrolysis by rat liver homogenate was identified by solubility and infrared spectrum to be the half-acid of malathion (Cook and Yip, 1958). These authors call the enzyme "malathionase." The enzyme hydrolyzed one of the carbethoxy groups:

$$(CH_3O)_2P(S)SCH(CH_2COOC_2H_5)COOC_2H_5 \xrightarrow{\text{carboxylesterase}} (CH_3O)_2P(S)SCH(CH_2COOC_2H_5)COOH$$

which was identified by Chen *et al.* (1969) to be the α-monoacid (structure above) based on nmr spectroscopy. The enzyme is widely distributed in mammals and has been found in the liver, kidney, sera, lung, spleen, and ilea of rats (Seume and O'Brien, 1960), mice, guinea pigs, and dogs (Murphy and DuBois, 1957). In each of the species studied the activity was always the highest in the liver. In general, the activity of the enzyme was low or absent in susceptible insects (Kojima, 1961), thus explaining in part the selective toxicity of the compound. The enzyme hydrolyzes triesters such as triacetin and tributyrin, but hydrolyzes the monocarbethoxy esters extremely slowly (Yip and Cook, 1959).

Main and Braid (1962) partially purified an enzyme from rat liver which hydrolyzed malathion. This enzyme hydrolyzed one of the carbethoxy groups and was characterized as a carboxylesterase or EC. 3.1.1.1 carboxylic-ester hydrolase.

A series of nonphosphorus esters was evaluated as substrates for the partially purified rat liver carboxylesterase. In Table IV the K_m and V_{max} are presented for mono- and diethyl carboxylic esters. The monoesters were good substrates for the enzyme, but the disubstituted esters bound much better as observed by K_m values. With the monoesters the K_m decreased from acetate to valerate. This also occurred with the malonate to adipate series. In both cases the longest chain-length compound in the "series" had the best K_m.

Table IV. Substrate Specificity of a Partially Purified Rat Liver Carboxylesterase[a]

Substrate	K_m mM	V_{max} μMoles/mg protein/min
Ethyl acetate	5.15	5.74
Ethyl propionate	2.86	19.6
Ethyl butyrate	3.9	42.6
Ethyl valerate	0.55	14.5
Diethyl malonate	3.57	37.0
Diethyl succinate	1.25	9.0
Diethyl glutarate	1.0	19.0
Diethyl adipate	0.47	19.2
Diethyl malate	1.0	4.86
Diethyl thiomalate	2.22[b]	10.0[b]

[a] Main and Dauterman (1970). [b] Activity determined at pH 6.0.

Table V. Substrate Specificity of a Partially Purified Rat Liver Carboxylesterase[a]

Substrate	R	K_m mM	V_{max} μMoles/mg protein/min
maleate	R		
HCCOR (O, //)	CH_3	12.5	1.1
HCCOR (=)	C_2H_5	8.6	10.0
HCCOR (=)	i-C_3H_7	2.4	0.26
HCCOR (\\ O)	n-C_3H_7	0.47	1.92
	n-C_4H_9	0.20	1.14
fumarate	R		
HCCOR (O, //)	CH_3	... unstable	...
HCCOR (=)	C_2H_5	1.17	19.0
ROCCH (\\ O)	i-C_3H_7	2.0	0.28
	n-C_3H_7	...	...
	n-C_4H_9	1.25	3.33

[a] Main and Dauterman (1970).

Addition of a hydroxyl or mercapto group as in malate and thiomalate has some effect on the K_m and on V_{max}.

Studies with dialkyl-substituted maleates and fumarates (Table V) indicated that the enzyme was able to hydrolyze both geometric isomers as well as the various alkyl substituents. In no case was there evidence that the enzyme was able to hydrolyze the second carbalkoxy group in any of the substrates evaluated. It has been reported that malathion diacid is excreted in the urine (Krueger and O'Brien, 1959). If one adds malathion monoacid to the system, the enzyme is unable to hydrolyze the remaining carbethoxy group. Therefore, the malathion diacid found in rat urine must be the result of self-hydrolysis of the monoacid on storage or on a column, or possibly another enzyme hydrolyzes the second carbethoxy group.

The studies were enlarged to use malathion homologs as substrates for the enzyme. The findings showed the carbutoxy analog to have the best K_m as well as the best V_{max} (Table VI). To evaluate the biological properties, the toxicological half-life was determined. This is the time necessary to reduce a given substrate concentration to half its initial value and includes a measure of affinity of the enzyme as well as the rate of reaction. The findings showed the carbutoxy compound had the shortest half-life and carbmethoxy the longest half-life. This correlated with the *in vivo* toxicity (Dauterman and Main, 1966).

Table VI. Activity of a Purified Preparation of Rat Liver Carboxylesterase Toward a Series of Malathion Homologs[a]

Compound	$K_m \pm$ SE (mM)	$V_{max} \pm$ SE (μmole/mg/min)	Relative enzymatic half-life [0.695 (K_m/V_{max})]
Carbomethoxy	0.506 ± 0.075	0.52 ± 0.04	0.676
Carbethoxy	0.179 ± 0.024	1.81 ± 0.12	0.069
Carb-*n*-propoxy	0.068 ± 0.018	2.46 ± 0.26	0.019
Carbisopropoxy	0.040 ± 0.007	0.28 ± 0.02	0.099
Carb-*n*-butoxy	0.022 ± 0.005	2.97 ± 0.24	0.005

[a] Dauterman and Main (1966).

Table VII. Activity of Carboxylesterase Toward Branched-Chain Analogs of Diethyl Malathion[a]

Compound	$K_m \pm$ SE (mM)	$V_{max} \pm$ SE (μmole/mg/min)	Relative enzymatic half-life [0.695 (K_m/V_{max})]
Succinate	0.205 ± 0.032	0.937 ± 0.043	0.152
Malonate	0.062 ± 0.012	0.338 ± 0.016	0.127
α-Glutarate	0.038 ± 0.006	0.525 ± 0.018	0.050
β-Glutarate	0.022 ± 0.003	0.953 ± 0.029	0.016

[a] Chiu *et al.* (1968).

Using the optical isomers of malathion as substrates, one finds that the *d*-isomer binds better (K_m 0.084 mM) to carboxylesterase than the *l*-isomer (K_m = 0.205 mM) (Hassan and Dauterman, 1968). The V_{max} was also slightly faster for the *d*-isomer. Clearly, the enzyme demonstrates specificity to both geometric isomers, as was demonstrated with the fumarates and maleates as well as with the optical isomers of malathion.

The studies were further enlarged to include malathion analogs where the leaving group was modified (Chiu *et al.*, 1968). Studies on the leaving groups show a wide variety in binding as well as reaction rates (Table VII). The symmetrical β-glutarate had the best K_m, as well as a good V_{max} value. With these compounds there was no correlation with the *in vivo* toxicity, since the α-glutarate compound was the most toxic compound in the series.

Summarizing the work we have done on the carboxylesterase and its substrate specificity, one can state that long-chained carbalkoxy compounds are better substrates than those with carbmethoxy groups. The *d*-isomer is preferred over *l*-isomer, and the fumarate-type compound, which allows rotation of the carbethoxy group, is preferred over maleate compounds. All of the substrates must be un-ionized at physiological pH in order for carboxylesterase to hydrolyze its substrates.

Dimethoate-Amidase System. Another *in vitro* system which operates in the mammalian liver is the dimethoate-amidase system. Dimethoate metabolism in vertebrates occurs almost exclusively in the liver (Uchida *et al.*, 1964). The importance of the cleavage of the C—N bond for selectivity was demonstrated by Krueger *et al.* (1960).

$$(CH_3O)_2P(S)SCH_2C(O)NHCH_3 \xrightarrow{\text{amidase}} (CH_3O)_2P(S)SCH_2COOH$$

Dimethoate degradation by liver homogenates of six vertebrates was investigated by Uchida and O'Brien (1967). A correlation between *in vitro* degradation and *in*

Table VIII. Subcellular Distribution of the Liver Amidase which Hydrolyzes Dimethoate[a]

Fraction	Activity % Rat	Sheep
Mitochondria	9	19
Microsomes	60	46
Supernatant	31	35

[a] Chen and Dauterman (1970b).

Table IX. The Effects of Cofactors on the Enzymatic Hydrolysis of Dimethoate[a]

	Activity (mμmoles of Dimethoate Hydrolyzed)
Control	73.6
NAD	79.1
NADH	79.1
NADP	79.1
NADPH	81.3
Co^{++}	81.4
Mg^{++}	77.4
Mn^{++}	79.4

The concentrations of nucleotides and metal ions are 1 μmole and 0.6 μmoles, respectively.

[a] Chen and Dauterman (1970b).

Table X. Substrate Specificity of Sheep Liver Amidase to *N*-Methylamides[a]

Substrate[b]	Relative Rate of Hydrolysis
N-methylformamide	0
N-methylacetamide	0
N-methylpropionamide	0
N-methylbutyramide	10.4
N-methylvaleramide	24.4
N-methylcaproamide	100
N-methylheptamide	86.5
N-methylcaprylamide	46.1
Dimethoate	54.9

[a] Chen and Dauterman (1970b). [b] Substrate concentration ($5 \times 10^{-3}M$).

vivo toxicity was found. Studies also indicated that the amidase which hydrolyzed dimethoate was primarily in the sheep liver microsomes (60%).

With these findings in mind, studies were undertaken by Chen and Yang (1969) to investigate the properties of the amidase and its substrate specificity. Initially, subcellular fractions of rat and sheep liver were isolated by differential centrifugation in order to study the distribution of the enzyme. The data in Table VIII indicate that the distribution is slightly different from the rat to the sheep, but the majority of the enzyme activity from both species is associated with the microsomal fraction. Our findings indicated that, based on wet weight, the sheep liver had approximately three times as much activity as the rat liver. Therefore, most of our studies were conducted with sheep liver preparations. Since differential centrifugation is a technique for purification, microsomal fraction was utilized as the starting material for purification. Repeating freezing and thawing solubilized the enzyme and resulted in a fivefold purification. At the present time, work is in progress to try to purify the enzyme further.

The possibility of an oxidative reaction mechanism similar to that reported by Lucier and Menzer (1970a) was investigated using certain cofactors (Table IX). The small increase in activity by the addition of the nucleotides suggests that this reaction is not associated with mixed-function oxidases. This reaction was not inhibited by carbon monoxide, thus also discounting oxygenases. A small increase in activity by the divalent cations also demonstrated that this enzyme was not an arylamidase, since these ions increase the activity of arylamidases.

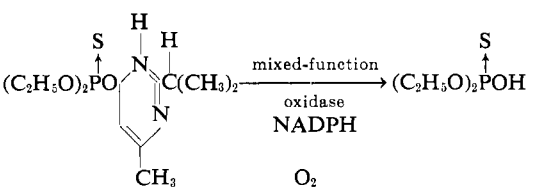
The hydrolysis of various substituted *N*-alkyl- and *N,N*-dialkylamides was studied using a colorimetric method based on the reaction of 2,4-dinitrofluorobenzene with the hydrolysis product (Chen and Dauterman, 1970a). The solubilized enzyme was unable to hydrolyze the short-chained *N*-methylamides (Table X). The best substrate in the series is the caproamide, and as one increases the chain length, the activity starts to decrease. The hydrolysis of various *N*-alkyl- and *N,N*-dialkylcaproamides was also investigated. The best binding occurred with the *N*-methyl compound which had a K_m of $6.64 \times 10^{-4}M$, and the binding decreased with an increase in chain length. The *N*-butylcaproamide had a K_m of $2.5 \times 10^{-3}M$. With the dialkylcaproamides only the dimethyl compound was hydrolyzed. The K_m of *N,N*-dimethylcaproamide was $1.76 \times 10^{-4}M$.

During these studies, the question arose whether the carboxylesterase, besides hydrolyzing malathion, could also hydrolyze dimethoate. Incubation of dimethoate with the carboxylesterase resulted in no hydrolysis of the insecticide.

With the preliminary work completed, further work is in progress on the purification of the enzyme as well as substrate specificity studies with dimethoate analogs.

Diazinon–Mixed-Function Oxidase System. A few years ago it was believed that organophosphates were hydrolyzed by phosphatases to either dialkyl phosphorothioic acid or diethyl phosphoric acid. Only recently has the work of Neal (1967a,b), Nakatsugawa and Dahm (1967), and Nakatsugawa *et al.* (1968, 1969) demonstrated that degradation of many phosphorothionate insecticides is the result of mixed-function oxidases.

In our laboratories, Yang (1970) has been studying the degradation of diazinon *in vitro*. Using various rat tissue homogenates, diazinon was hydrolyzed when NADPH was added to liver homogenates. All other tissues were inactive (Yang *et al.*, 1969). Subsequent subcellular fractionation showed the reaction to be localized in the rat liver microsomes. Studying the effect of various additives showed that NADPH was required as well as oxygen. The addition of carbon monoxide and nitrogen decreased the activity of microsomal degradation of diazinon. The carbon monoxide inhibition demonstrated that cytochrome P_{450} was involved in this reaction. These necessary requirements classified this reaction as a mixed-function oxidase.



The products isolated when diazinon was incubated with microsomes and NADPH were diethyl phosphorothioic acid and diethyl phosphoric acid. Diazoxon was isolated in extremely small amounts. Studies on the diazoxon-degradation showed the enzyme also to be localized in the microsomes (Table XI). However, this reaction is not dependent on oxygen or NADPH, and therefore is not a mixed-function oxidase. Clearly, it is a hydrolase with the ability to degrade the phosphate. The indications are that the mixed-function

Table XI. Activity of Diazoxon-Degrading Enzyme in Rat Liver Fraction[a]

Fraction	Degradation μmoles/30 min/10 mg of liver with NADPH	without NADPH
Nuclei	2.2	2.5
Mitochondria	1.4	1.6
Microsomes	7.0	7.8
Soluble fraction	0.3	0.2

[a] Yang (1970).

oxidase oxidatively degrades diazinon to diethyl phosphorothioic acid and also desulfurates diazinon to diazoxon. The diazoxon which is formed is rapidly degraded by the hydrolase in the rat liver microsomes. The housefly microsomal system functions slightly differently (Hodgson, 1970). The mixed-function oxidase system of the housefly oxidatively degrades both diazinon and diazoxon, but diazinon is the preferred substrate (Yang, 1970).

Each of the *in vitro* systems discussed allows the mammal to detoxify the parent organophosphate. Generally, the titer of the specific enzyme is low or the enzyme is absent in insects, allowing the mammal a degree of selectivity.

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