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METABOLISM OF ORGANOPHOSPHORUS INSECTICIDES—XI

METABOLIC FATE OF DIMETHOATE IN THE RAT

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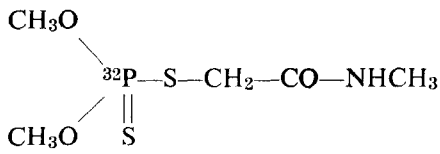
Abstract—The metabolic fate of Dimethoate in the rat has been investigated *in vivo* and *in vitro*, using ^{32}P-Dimethoate and ^{14}C-Dimethoate labelled at two different sites. After 24 hr, about 60 per cent of the administered dose (30 mg/kg) was eliminated in the urine and the expired air. Oxidation of Dimethoate to Dimethoxon is believed to occur *in vivo*. A major hydrolytic pathway involves cleavage of the C–N bond of Dimethoate (and probably also of Dimethoxon) by a carboxy amidase. The liberated methylamine is oxidatively demethylated mainly to CO_2 and a trace of formate. A second major pathway involves an esterase action on the S–C bond of the parent insecticide (and probably also of Dimethoxon). About 80 per cent of the non-phosphorus moiety is believed to be eliminated in the urine, without further modification, as glucuronide. The phosphorus moiety is excreted either as such (Dimethylphosphorothioic and Dimethylphosphorodithioic acids) or undergoes further modification to metabolites like Dimethylphosphoric acid, Monomethylphosphoric acid and Thiophosphoric acid. Methanol released from the latter two substances is transformed, almost quantitatively to CO_2 and formate.

The kinetic data indicate that Dimethoxon is 75–100 times as potent as Dimethoate in inhibiting rat brain acetylcholinesterase; suggesting that Dimethoxon plays the dominant role in mammalian toxicity. The irreversible reaction of Dimethoxon with acetylcholinesterase was found to be of bimolecular nature ($k = 1{\cdot}65 \times 10^{-3}$ mole^{-1} min^{-1}). The reaction of Dimethoate with the enzyme is partially reversible.

O,O-DIMETHYL S-(*N*-METHYLCARBAMOYL METHYL) PHOSPHORODITHIOATE (Dimethoate) posseses a wide spectrum of activity, both as contact and as systemic insecticide. It was found effective for the control of a large number of phytophagous insects.[1] The high selectivity of Dimethoate has been demonstrated by a number of authors.[2–4] This selectivity oriented research on the metabolic fate of the insecticide in plants,[5–7] and insects.[8–10] Degradation of Dimethoate was also studied in mammals.[3,11,12]



I

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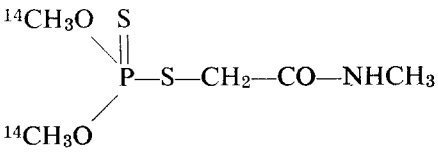
In order to gain a closer understanding of the mechanism of detoxification, two types of radioactive insecticide—in addition to the ^{32}P-labelled compound I—have been used. These are ^{14}C-Dimethoate II and ^{14}C-Dimethoate III.



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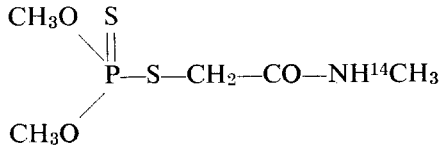


III

In an attempt to assess the potency of the P=O analogue of Dimethoate (Dimethoxon) as an anticholinesterase agent, the kinetics of its reaction with rat-brain acetylcholinesterase have been studied.

MATERIALS AND METHODS

Dimethoate I was prepared according to Zayed *et al.*,[10] and possessed a specific activity of 5×10^5 cpm/mg. Dimethoate II and III were obtained by the procedure of Zayed *et al.*,[13] and had a specific activity of $3{\cdot}6 \times 10^4$ and $2{\cdot}4 \times 10^4$ cpm/mg respectively.

Throughout this investigation, albino rats of both sexes, weighing 100–120 g and maintained on a stock diet were used. Radioactive Dimethoate in distilled water was injected i.p. at a single dosage of 30 mg/kg.

Experiments with Dimethoate I

In vivo. Urine was collected 24 hr after injecting the radio-compound, extracted five times with chloroform and concentrated under vacuum at room temperature. The aqueous concentrate was applied on an anion exchanger (Dowex 1-X8, Cl^-, 100–200 mesh, 15×1 cm), and washed with water till no more radioactivity was detectable in the eluate. The acidic metabolites were eluted from the column as described by Zayed *et al.*[10] For identification purposes, samples of the chloroform extract and the eluted fractions were analyzed by radiopaper chromatography.

In vitro. Five milliliters of 10% rat liver homogenate in phosphate buffer (0·2 M), pH 7·4 were incubated at 37° for 5 hr with 18 mg Dimethoate I (9×10^6 cpm). At the end of the incubation period, the reaction mixture was extracted five times with chloroform, using 5 ml at a time; the emulsions were broken by centrifugation. The aqueous layer was applied on an anion exchanger for separation of the acidic metabolites, as described above. Identification of metabolites was achieved by radiopaper chromatography.

Experiments with Dimethoate II and III

Urine from Dimethoate II-treated rats was analyzed in a manner similar to that described for Dimethoate I. For the collection of respiratory $^{14}CO_2$, metabolic cages have been used.[14] The ^{14}C-activity was trapped by 1N sodium hydroxide solution, and determined as $Ba^{14}CO_3$. ^{14}C-activity in the urine or eluted fractions was determined (after the solution was dried over P_2O_5) according to the procedure described by Aronoff,[15] using Van Slyke–Folch reagent.[16] For isolation and determination of ^{14}C-formate in the urine, the inverse isotope dilution technique was adopted. The

procedure is that essentially described by Hassan and Zayed.[17] Characterization of the isolated radioformate was achieved by radiopaper chromatography. Metabolites from the *in vitro* experiments (with Dimethoate II) were analyzed by column fractionation and paper chromatography.

Paper chromatography

The ascending chromatographic technique has been adopted using Schleicher and Schüll paper 2043b. The following seven solvent systems were used:

A. Papers were impregnated with 0·2 M potassium acetate buffer (pH 3·6) and developed with isobutanol : 0·2 M acetate buffer (pH 3·6), 5 : 1.
B. Acetonitrile : water : ammonia (40 : 9 : 1)
C. Isopropanol : water : ammonia (75 : 24 : 1)
D. Isopropanol : ammonia (75 : 25)
E. *n*-Butanol : pyridine : water (12 : 8 : 6)
F. *n*-Butanol : acetic acid : water (4 : 1 : 5)
G. *n*-Butanol : acetic acid : water (4 : 1 : 5); papers impregnated with 0·2% sodium acetate solution.

The chromatograms were developed for 4–16 hr (according to system), and then left to dry in the air. The chromatograms were assayed radiometrically, and the spots were made visible by spraying with Hanes–Isherwood reagent.[18] Phosphorothioate compounds were located by spraying the chromatograms with 2% cupric chloride solution, and then with 0·5% potassium ferricyanide solution; where red-brown spots appeared on a yellow-green background.[19]

Radiomeasurements

^{32}P-activity measurements of the urine and the fractions eluted from the anion exchanger were carried out in solution (3 ml) using a Geiger counter with an end-window Tracerlab G-M tube. Radiometric assay of the paper chromatograms was made with Frieske & Hoepfner radioscanner. Sample counting was performed under uniform geometrical conditions, and measurements were corrected for decay and background. No allowance has been made for self-absorption.

^{14}C-activity was determined as $Ba^{14}CO_3$ and corrected for background and self-absorption.

Kinetic studies

Non-labelled Dimethoxon and Dimethoate were used for the kinetic studies. Rat-brain homogenate served as a source of acetylcholinesterase and the enzyme was assayed according to the method of Hestrin.[20] The reaction mixture had the following composition: 0·9 ml 0·2 M phosphate buffer, pH 7·2; 0·3 ml 0·4 M magnesium chloride solution; 0·3 ml 1·0 M sodium chloride solution; 0·5 ml 10% rat brain homogenate in isotonic KCl; 0·5 ml distilled water or variable concentrations of the inhibitor in distilled water (final concentration 5×10^{-6}–5×10^{-3} M); 0·5 ml 24 mM acetylcholine chloride in 0·001 M sodium acetate, to be added after the preincubation period (final concentration 4 mM). The period of assay of the enzyme activity was 30 min.

RESULTS

Dimethoate I

After 24 hr, the ^{32}P-activity recovered from the urine as hydrolytic products con-

tributed 55–63 per cent of the administered dose. The results of a typical experiment are given in Table 1. The chloroform extract from urine showed the presence of small amounts of Dimethoate (4–7 per cent of the administered dose); as the only organic-soluble ^{32}P-substance. The acidic ^{32}P-metabolites were identified by radiopaper chromatography (Table 2).

The *in vitro* degradation of the pesticide was studied by incubating Dimethoate I

TABLE 1. TYPES AND PERCENTAGES OF THE ACIDIC ^{32}P-LABELLED METABOLITES RECOVERED FROM THE ANION EXCHANGER

^{32}P-activity eluted with	cpm*	Percentage recovery (total fractions = 100%)	Metabolites identified in the eluate	% metabolite†
HCl, pH 1	467,000	26·9	Monomethylphosphate	5
			Dimethylphosphate	18
			Thiophosphoric acid	4
NHCl : methanol (1 : 3)	1120,000	65·6	Dimethoate carboxylic acid	32
			Dimethylphosphorothioic acid	30
			Monomethylphosphate	1
			Dimethylphosphate	3
NHCl : acetone (1 : 3)	127,000	7·5	Dimethylphosphorodithioic acid	7

* Administered dose 6 mg = 3×10^6 cpm.
† Percentages of metabolites are estimated from paper chromatographic analysis (100% = total hydrolytic products).

TABLE 2. R_f VALUES OF DIMETHOATE AND ITS METABOLITES

Substance	System* A	B	C	D	E	F	G
Dimethoate	0·86	0·99	0·95	0·93	0·93		
Dimethoxon	0·72	0·85	0·87	0·83	0·85		
Dimethoate carboxylic acid	0·50	0·59	0·69	0·71	0·60		
Dimethylphosphorothioic acid	0·06	0·35	0·68	0·63	0·51		
Dimethylphosphorodithioic acid	0·14	0·50	0·71	0·69	0·62		
Dimethylphosphoric acid	0·04	0·24	0·57	0·50	0·17		
Monomethylphosphoric acid	0·45	0·05	0·11	0·04	0·04		
Thiophosphoric acid	0·28	0·10	0·03	0·02	0·01		
Formate			0·53			0·50	0·50
Conjugated metabolite (from Dimethoate III)			0·30			0·44	0·34

* Experimental conditions are described under "methods".

with liver homogenate. Under the prevailing experimental conditions, the liver degraded 3·7–5·8 per cent of the initial amount of the insecticide. Two main metabolites were identified by paper chromatography. These are Dimethoate carboxylic acid and Dimethylphosphorodithioic acid. The chloroform extract of the reaction mixture contained only Dimethoate.

Dimethoate II

In a typical experiment following the injection of 9 mg Dimethoate II, 51·8 per cent

of the administered radioactivity could be recovered after 24 hr. $^{14}CO_2$ in the expired air contributed 4·4 per cent of the initial dose. ^{14}C-formate recovered from the urine constituted 4·2 per cent. Radioactive acidic metabolites in the urine (after removal of ^{14}C-formate) accounted for the major part of the degradation products (43·2 per cent). The identification of these metabolites was achieved by fractionation on an anion exchanger, followed by paper chromatography of the different fractions. The urinary hydrolytic metabolites are essentially the same as those obtained with Dimethoate I (Table 3). Also, the chloroform extract of the urine was found to contain a small amount of the parent insecticide.

Experiments *in vitro* with Dimethoate II and liver homogenate confirmed the results obtained with Dimethoate I.

TABLE 3. URINARY ^{14}C-METABOLITES (AFTER REMOVAL OF ^{14}C-FORMATE) FROM DIMETHOATE II

^{14}C-activity eluted with	cpm*	Percentage recovery (total fractions = 100%)	Metabolites identified in the eluate	% metabolite†
HCl, pH 1	48,000	34·3	Monomethylphosphate	4
			Dimethylphosphate	30
N–HCl : methanol (1 : 3)	86,000	61·4	Dimethoate carboxylic acid	38
			Dimethylphosphorothioic acid	21
			Traces of mono-and-Di-methylphosphates	2
N–CHl : acetone (1 : 3)	6000	4·3	Dimethylphosphorodithioic acid	5

* Administered dose 9 mg = $3·24 \times 10^5$ cpm.
† Percentages of metabolites are estimated from paper chromatographic studies (100% = total hydrolytic products).

Dimethoate III

Following the administration of this isotope, radioactive CO_2 was eliminated in the expired air (15–18 per cent of the initial dose, for the first 24 hr). The formation of $^{14}CO_2$ (as a result of carboxyamidase action and consequent oxidation of the amine) starts almost instantaneously, and within 6 hr the major part (over 90 per cent) has been eliminated. After 6 hr, a remarkable decrease in the elimination rate is observed. ^{14}C-formate in the urine accounted for only 0·2–0·4 per cent of the injected dose.

The 24 hr-urinary ^{14}C-metabolites constituted 39–45 per cent of the initial dose. About 80 per cent of the radioactivity was found to be incorporated into one metabolite which does not contain phosphorus or sulfur, and seems to be excreted in a conjugated form (Table 2). Characterization of this metabolite followed the same procedure as described by Zayed *et al.*[13] The conjugating material was found to be glucuronic acid.

Kinetic studies

The rate of the reaction of Dimethoxon (P=P analogue of Dimethoate) with rat-brain acetylcholinesterase has been studied according to Aldridge.[21] The plot of

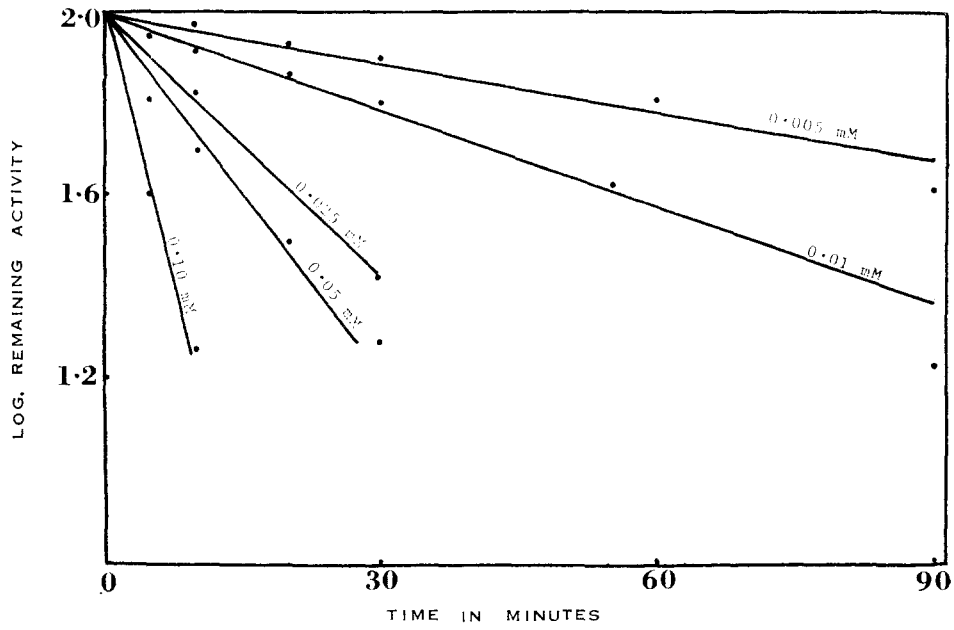


FIG. 1. Rate of inhibition of rat-brain acetylcholinesterase by Dimethoxon. Inhibitor concentration (millimoles/liter) is shown against each curve.

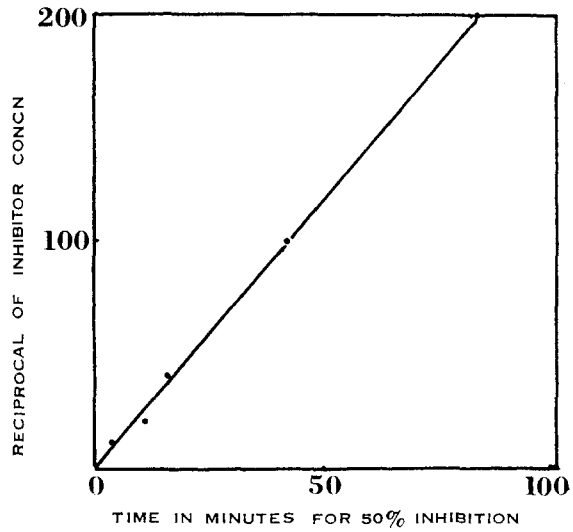


FIG. 2. Inverse proportionality of inhibitor concentration to time necessary for 50 per cent inhibition (data taken from Fig. 1).

logarithm of percentage of remaining activity against time gave a straight line for each inhibitor concentration; thus showing the characteristics of a unimolecular reaction (Fig. 1). The type of reaction of Dimethoxon with the enzyme has been found to be bimolecular (Fig. 2). The velocity constant (k) for the bimolecular reaction has been calculated to be $1{\cdot}65 \times 10^{-3}$ mole^{-1} min^{-1}.

Dimethoxon (5×10^{-5} M) (added 30 sec after the addition of acetylcholine) caused only a slight enzyme inhibition (Table 4). Concentrations of acetylcholine as high as 8 mM prevented the inhibition completely; at least during the first 30 min. Removal of the free inhibitor, by washing the enzyme protein with buffer, did not result in recovery of the enzyme activity (Table 4).

TABLE 4. PROTECTION OF ACETYLCHOLINESTERASE FROM INHIBITION BY SUBSTRATE AND EFFECT OF WASHING ON THE ENZYME RECOVERY

Treatment	% inhibition by Dimethoxon*	Dimethoate*
I added to E, 30 sec before S	9	30
S added to E, 30 sec before I	3	26
I added to E, 30 min before S	81	82
I incubated with E for 30 min and washed once with buffer	85	67
I incubated with E for 30 min and washed twice with buffer	82	61

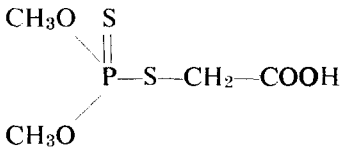
I = Inhibitor (Dimethoxon or Dimethoate)
E = Enzyme (Brain homogenate)
S = Substrate (Acetylcholine)
* Dimethoxon concentration = 5×10^{-5} M; Dimethoate concentration = 5×10^{-3} M.

Similar studies have been carried out to investigate the reaction of the parent substance, Dimethoate, with acetylcholinesterase. The plot of logarithm of percentage of remaining activity versus time did not give straigh tlines as the P=O analogue. The last part of the curve tends to level off (60–150 min). Acetylcholine was found capable of partial protection of the enzyme (Table 4). Washing the free inhibitor resulted in partial recovery of the enzyme activity (Table 4).

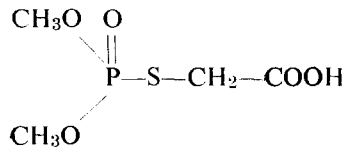
From kinetic studies of the Dimethoxon–acetylcholinesterase system, an I_{50} value of $1{\cdot}2 \times 10^{-5}$ M has been obtained. The corresponding value for Dimethoate was calculated to be $9{\cdot}0 \times 10^{-4}$ M (I + E for 30 min in absence of S).

DISCUSSION

The data presented in this investigation show that Dimethoate is fairly rapidly metabolized in the rat. About 60 per cent of the administered dose was eliminated, after 24 hr, in the urine and the expired air. The Dimethoate molecule suffers oxidation and hydrolysis to give a variety of metabolites. Oxidation to the more toxic P=O analogue is assumed to occur *in vivo*.[3] Cleavage of the C–N bond by a carboxyamidase constitutes a major hydrolytic pathway to produce the carboxy derivative IV.



IV

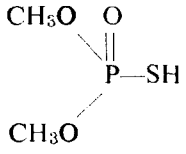


V

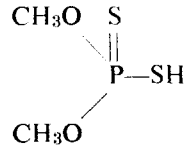
This may partially suffer further hydrolysis at the S–C bond.[3] The corresponding carboxy derivative of Dimethoxon V could not be detected in urine. The liberated methylamine moiety is oxidatively demethylated, almost quantitatively to CO_2 which is eliminated in the expired air and a small amount of formate which is excreted in the urine. The oxidation pathway is presumably as follows:

$$NH_2{}^{14}CH_3 \rightarrow H^{14}CHO \rightarrow H^{14}COOH \rightarrow {}^{14}CO_2$$

A second hydrolytic pathway involves an esterase action on the S–C bond of Dimethoate (or Dimethoxon). The phosphorus moiety may be eliminated without further changes, i.e. as *O,O*-Dimethylphosphorothioic acid VI and *O,O*-Dimethylphosphorodithioic acid VII. On the other hand,

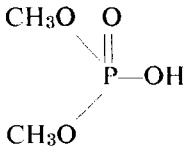


VI

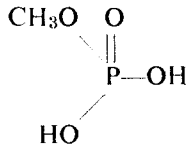


VII

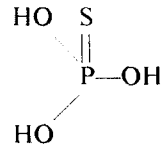
the phosphorus moiety may suffer further modification to metabolites like Dimethylphosphoric acid VIII, Monomethylphosphoric acid IX and Thiophosphoric acid X. The fate of the methyl groups released by VIII and IX could be



VIII

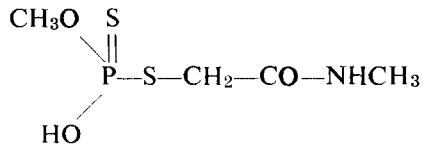


IX



X

traced using Dimethoate II. Apparently, a phosphatase action is involved to liberate methanol which is oxidized to ^{14}C-formate and $^{14}CO_2$.[17] It is probable that IX and X are produced by a phosphatase action on the intact Dimethoate molecule (or Dimethoxon for IX), followed by cleavage of the S–C bond. In this connection, Dauterman *et al.*[3] were able to detect a small amount of Desmethyl Dimethoate XI in the rat urine.



XI

The major part of the non-phosphorus moiety (80 per cent), resulting from hydrolysis of the S–C bond conjugates with glucuronic acid and is eliminated in the urine. This substance is also excreted as a major metabolite by the cotton leaf worm.[13] From degradation and chromatographic studies,[13] this product was given the structure XII. A minor part of the non-phosphorus moiety probably undergoes a modification of the methyl group.

Glucuronic acid—O—CH_2—CO—$NHCH_3$

XII

The *in vitro* experiments reveal two main types of hydrolysis in the liver. One involves the amidase which is presumably concentrated in the microsomal faction.[22] The other type produces VII after cleavage of the S–C bond.

From kinetic studies of the acetylcholinesterase–Dimethoxon system it becomes evident that the inhibitor is a powerful anticholinesterase agent. The progressive nature of inhibition suggests an irreversible inactivation of the enzyme, and the effectiveness of Dimethoxon is expressed by a velocity constant k. The irreversible inhibition of the enzyme also suggests that brain-acetylcholinesterase is not concerned with the detoxification of Dimethoxon to any significant extent. The irreversible nature of the reaction is well confirmed by failure of the washing technique in restoring the enzyme activity. Studies concerned with the protection of the enzyme by substrate reveal a competitive type of effect. The competition between the substrate and Dimethoxon for the enzyme indicates that the organophosphorus compound attaches itself to the substrate-binding group in the active center.

The results obtained from the acetylcholinesterase-Dimethoate system suggests that the reaction is partially reversible. In this respect, Dimethoate behaves similar to the carbamate insecticide Carbaryl.[23]

By comparing the data, it could be estimated that Dimethoxon is 75–100 times as potent as Dimethoate, in inhibiting acetylcholinesterase. This would indicate that "Dimethoate as such" probably contributes only a minor part in mammalian toxicity, and that its P=O analogue is the main toxic agent. In this connection, it has been shown that pretreatment of mice with sodium phenobarbital increased the toxicity of Dimethoate more than three times.[24] This is because the hepatic microsomal enzyme(s) responsible for the conversion of Dimethoate to its P=O analogue, has been induced.

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