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# METABOLISM OF ORGANOPHOSPHORUS INSECTICIDES—XII*

## DEGRADATION OF $^{32}$P-MALATHION IN THE ADULT LARVA OF THE COTTON LEAF WORM

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**Abstract**—The metabolism of Malathion in the adult larva of the cotton leaf worm (*Spodoptera littoralis*) has been investigated *in vivo* using $^{32}$P-labelled insecticide. After 24 hr, about 90 per cent of the applied dose is metabolized. The oxidation of P=S to P=O and the phosphatase action represent the most important degradation pathways of the insecticide. Eight metabolites have been isolated and identified.

MALATHION (I)† is an important and widely used pesticide because of its wide spectrum of activity and its extremely low mammalian toxicity.[1] It has found applications in the fields of plant protection and in veterinary medicine. In Egypt, Malathion has been accepted for controlling pests on vegetables and field crops. It seems of interest therefore to study the metabolism of this important insecticide in the cotton leaf worm, *Spodoptera littoralis*, a serious pest in Egypt.

A number of investigations on the biochemical degradation of Malathion in insects and animals have been recently reported in the literature. Its high insect toxicity has been attributed to the activation to the more toxic oxygen analogue, Malaoxon (II).[2] The detoxication of Malathion in insects, mammals and plant tissues was reported to take place mainly through the action of carboxyesterase(s) and phosphatase(s).[3–16]

The present work was undertaken to gain insight into the mode of action and metabolic fate of $^{32}$P-Malathion in larvae of *Spodoptrea littoralis*. The metabolism of Malathion in the rat was carried out for comparison purposes.

## MATERIALS AND METHODS

O,O-*Dimethyl* S-(1,2-bis-*carboethoxy*) *ethyl phosphorodithioate.* $^{32}P_2S_5$ Used for the preparation of radioactive $^{32}$P-Malathion has been prepared by an exchange reaction between non-labelled $P_2S_5$ (0·25 g) and $^{32}$P-orthophosphate (20 mCi) in an atmosphere of $CO_2$ as described by Casida.[17]

To a suspension of the product in 10 ml dry benzene, a solution of 0·2 ml anhydrous methanol dissolved in 5 ml dry benzene was added dropwise while stirring at room

* For part XI, cf. S. M. A. D. ZAYED, A. HASSAN, I. M. I. FAKHR and M. R. E. BAHIG, *Biochem. Pharmac.* **18**, 2429 (1969).

† All roman numerals refer to compounds depicted in Fig. 4.

temperature. The contents were refluxed at 75–80° until all the $^{32}P_2S_5$ had reacted. The temperature was dropped to 50° and 0·425 ml/diethyl maleate in 5 ml/dry benzene were added. The mixture was then refluxed at 75–80° with stirring for 4 hr. After removal of benzene, the residue was dissolved in chloroform, washed with an equal amount of cold 10% sodium carbonate solution and then with cold saturated solution of sodium chloride. The chloroform layer was dried over anhydrous sodium sulphate and then evaporated in a vacuum. The yield of $^{32}$P-Malathion was 530 mg (about 72 per cent) with an initial specific activity of 0·025 mCi/mg ($3·6 \times 10^5$ cpm/mg). Examination by radio–paper chromatography showed that the product was 99% Malathion.

*Malathion mono-* (IV) *and diacids* (V). The mixture of α- and β-monoacids [*O,O*-dimethyl-*S*-(1-carboethoxy-2-carboxy(ethyl phosphorodithioate], was prepared according to Chen *et al.*[18] from *O,O*-dimethyl phosphorodithioc acid and maleic anhydride. The isomers could not be separated from each other chromatographically using the techniques available.

*O,O*-Dimethyl *S*-(1,2-*bis*-carboxy)-ethyl phosphorodithioate was prepared from *O,O*-dimethyl phosphorodithioic acid and maleic anhydride according to March *et al.*[9]

*Distribution.* For each gram of insect, 200 μg of the insecticide ($7·2 \times 10^4$ cpm) in 20 μl acetone were uniformly applied on the dorsal side. At specified time intervals after treatment, the unabsorbed insecticide was removed by acetone. Samples of the hemolymph, gut and fat were freshly weighed and measured for their radioactivity.

*Experiments* in vivo

*Spodoptera littoralis.* In these experiments, healthy adult larvae (5th–6th instars) of almost the same size and weight were used.

$^{32}$P-Malathion, dissolved in acetone was applied topically at a dose level of 500 μg (180,690 cpm)/gram insect. The larvae were left in a beaker containing a little sawdust for 24 hr, after which the excreta were exhaustively extracted with distilled water. To extract the remaining activity in the insects, they were homogenised with a least amount of acetone–water (19:1) and homogenates were chilled at −10° and centrifuged. From the supernatant, acetone was driven off under nitrogen atmosphere. The rest was extracted with water and then with chloroform.

The combined aqueous extracts were then shaken several times with chloroform. Both the chloroform extracts and the aqueous layer were analysed for possible metabolites.

*Chloroform extracts.* The combined chloroform extracts were concentrated under reduced pressure at room temperature. Samples of the chloroform solution were used for paper chromatographic analysis in different solvent systems (Table 1). The chloroform concentrate was also analysed by partition chromatography on aluminum oxide according to Krueger and O'Brien,[2] using benzene and chloroform for elution.

*Aqueous extracts.* The aqueous extracts were concentrated to about 10 ml under reduced pressure at room temperature to avoid any possible decomposition of the metabolites. For the resolution of the water soluble metabolites, the water concentrate was applied on an anion exchanger column [Dowex 1-X8, $Cl^-$, 100–200 mesh (15 × 1 cm)]. The column was first washed with 200 ml distilled water and the acidic metabolites were isolated by gradient elution using the following series of solvent systems in succession: (A) 0·01 N HCl/0·1 N HCl; (B) 0·01 N HCl + methanol

TABLE 1. PERCENTAGE AND $R_f$ VALUES OF MALATHION AND ITS DEGRADATION PRODUCTS IN SEVERAL SOLVENT SYSTEMS

| Peak | Substance | Percentage* | | System† | | |
|---|---|---|---|---|---|---|
| | | Larva | Rat | A | B | C |
| | Chloroform layer | | | | | |
| | Malathion | | | 0·96 | 0·94 | 0·92 |
| | Malaoxon | 25·2 | 1 | 0·81 | 0·89 | 0·86 |
| Aqueous layer | | | | | | |
| 1 + 2 | Phosphoric acid‡ | 11·0 | | 0·00 | 0·00 | 0·03 |
| 1 + 2 | Thiophosphoric acid‡ | 18·0 | 4·5 | 0·04 | 0·03 | 0·14 |
| 1 + 2 | Monomethyl phosphate‡ | 7·2 | 5·3 | 0·06 | 0·08 | 0·25 |
| 3 | Dimethyl phosphate | 15·0 | 18·1 | 0·17 | 0·62 | 0·41 |
| 4 | Malathion monoacid§ | 11·2 | 43·7 | 0·33 | 0·38 | 0·37 |
| 5 | Malathion diacid‖ | 3·0 | 7·5 | 0·13 | 0·31 | 0·30 |
| 6 | *O,O*-Dimethyl phosphorothioate | 6·9 | 11·7 | 0·42 | 0·68 | 0·64 |
| 7 | *O,O*-Dimethyl phosphorodithioate | 2·5 | 5·0 | 0·51 | 0·79 | 0·81 |

* Total metabolites = 100 per cent.

† For paper chromatography, Whatman No. 1 paper was used at 30°.

(A) Acetonitrile–water–ammonia (85:14:1)[3,22]

(B) Iso-propanol–water–ammonia (75:24:1)[21,22]

(C) *n*-Butanol–*n*-butyric acid–water (2:2:1)[1]

‡ The ion exchange system used did not adequately separate phosphoric, thiophosphoric and monomethyl phosphoric acids, the identification and percentages of these substances were carried out by paper chromatography.

§ *O,O*-Dimethyl *S*-(1-carboethoxy-2-carboxy) ethyl phosphorodithioate.

‖ *O,O*-Dimethyl *S*-(1,2-biscarboxy)ethyl phosphorodithioate.

(1:4)/0·1 N HCl + methanol (1:4); (C) 0·1 N HCl + methanol (1:3)/1 N HCl + methanol (1:3); (D) 1 N HCl + acetone (1:3)/concn HCl + water + acetone (1:1:6).

Three-ml fractions were measured for their $^{32}P$-activity, for their total phosphorus content,[19] then analysed by paper chromatography.

*Rats.* In these experiments, albino rats of both sexes weighing 100–200 g and maintained on a stock diet were used.

Radioactive Malathion was dissolved in water as a suspension using a detergent (1%) and injected intraperitoneally (30 mg/kg). Urine was collected for 24 hr using a metabolic cage, it was extracted five times with chloroform and then concentrated under vacuum. Samples of the chloroform extract were analysed by paper chromatography. The aqueous concentrate was applied on an anion exchanger column and treated as in the case of the larvae.

For identification purposes, samples of the eluted fractions were analysed by radio-paper chromatography (Table 1).

Spots were made visible by spraying with Hanes–Isherwood reagent.[20] To locate the phosphorothioate compounds, the papers were sprayed with a 2% solution of cupric chloride and then with a 0·5% solution of potassium ferricyanide, where red-brown spots on a yellow-green background[21] were obtained.

*Radio-measurements.* $^{32}P$-activity eluted from the anion exchanger was determined in solution. All measurements were carried out in an end window Tracerlab G-M tube

under uniform geometrical conditions. The data were corrected for decay and background and no allowance was made for self absorption.

Radiometric assay for paper chromatograms was carried out with a similar device.

## RESULTS

*Distribution.* The distribution of $^{32}$P-activity among hemolymph, gut and fat in *Spodoptera littoralis* larva after topical application of $^{32}$P-Malathion is illustrated in Fig. 1. After 1 hr, the larva was found to contain 17·8 per cent of the applied radiodose. The radioactivity in the hemolymph and the gut reached its maximum after 1 hr and

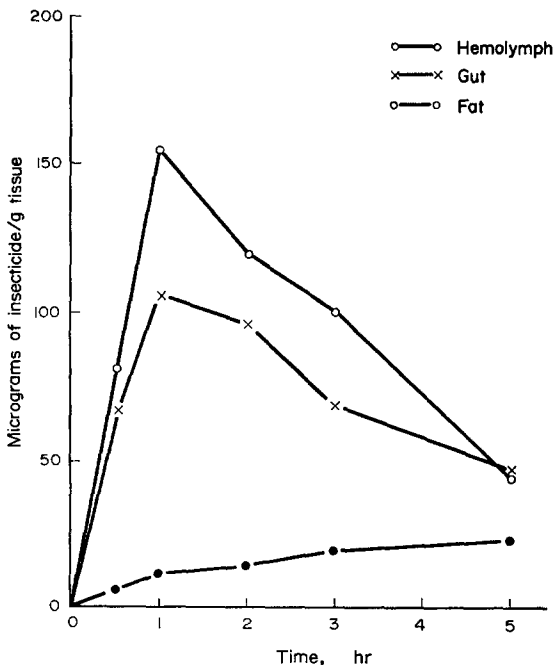


FIG. 1. Uptake of $^{32}$P-activity (micrograms of Malathion) in different organs of the larva of *Spodoptera littoralis* as a function of time.

decreased gradually with time. The fat, on the other hand, showed a definite progressive increase in radioactivity with time.

*Metabolism* in vivo. *Spodoptera littoralis.* For this experiment, a pool of 40 g insects was used, the applied $^{32}$P-Malathion was 20 mg ($7{\cdot}2 \times 10^6$ cpm). After 24 hr, 28·2 per cent of the applied $^{32}$P-activity ($2{\cdot}0 \times 10^6$ cpm) could be recovered in the chloroform extract. From several chromatograms and from partition chromatography on aluminum oxide column, it has been estimated that unchanged Malathion contributed to about 25 per cent of the chloroform extractable activity ($5 \times 10^5$ cpm).

The combined aqueous extracts contained 62·5 per cent of the applied dose (4·5 × $10^6$ cpm), and the $^{32}$P-activity remaining in the larvae and the saw dust was about 7·5 per cent of the applied dose (5·4 × $10^5$ cpm), thus demonstrating an excellent recovery of the radioactivity (about 98·2 per cent).

*Chloroform layer.* Radio–paper chromatographic analysis as well as partition chromatography of the chloroform extractable radioactivity on aluminum oxide,[2] showed the presence of unchanged Malathion and the oxygen analogue (Malaoxon II). The former was eluted with benzene and the latter with chloroform. The $R_f$ values of Malathion and some of its degradation products in different solvent systems are listed in Table 1.

*Aqueous extracts.* Figure 2 shows an elution curve of the acidic $^{32}$P-labelled metabolites using hydrochloric acid and mixtures of hydrochloric acid–methanol or acetone for elution. The identity and percentage of the acidic $^{32}$P-labelled substances recovered from the anion exchanger are shown in Table 1.

Radio–paper chromatography of the water soluble hydrolytic products confirmed the presence of eight metabolites given in Table 1. Authentic substances were run alongside for identification purposes.

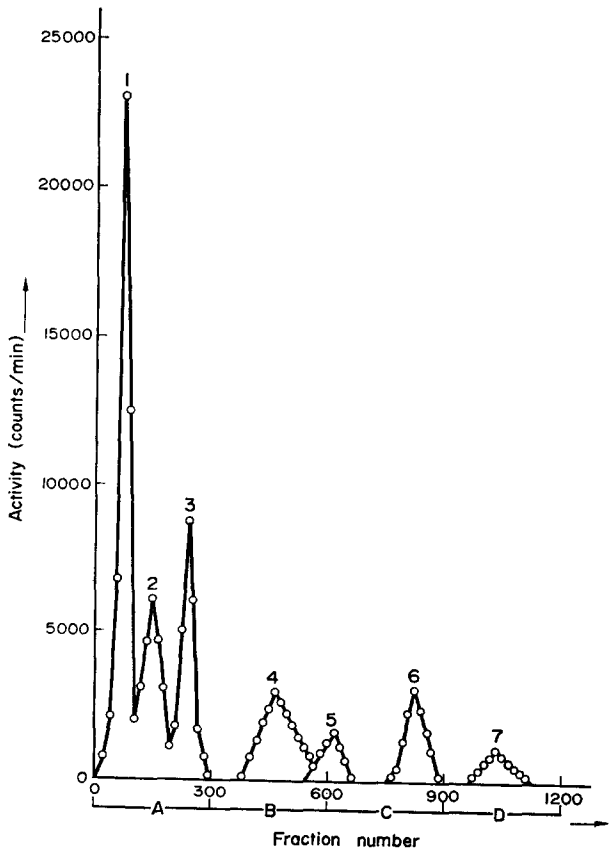


FIG. 2. Fractionation of the water soluble metabolites in *Spodoptera* larva after application of $^{32}$P-Malathion.

*Rats.* After 24 hr, 1–2 per cent of the administered $^{32}P$-activity could be recovered in the chloroform extract of the urine as residual Malathion and about 1 per cent only as Malaoxon. The aqueous layer contained 65–70 per cent of the administered $^{32}P$-activity. The water soluble metabolites and their percentages are shown in Table 1.

## DISCUSSION

Distribution studies showed that Malathion $^{32}P$-activity did not tend to accumulate in high concentrations within the larva.

After 1 hr from topical application, the larva was found to contain about 18 per cent of the applied radiodose. This may indicate effective excretion of the insecticide and/or metabolites by the adult *Spodoptera* larva.

From metabolism experiments it was found that in *Spodoptera* larva, about 93 per cent of the topically applied dose is metabolized during 24 hr. The products of metabolism suggest that the Malathion molecule undergoes two main metabolic pathways in both larva and rat. One consists of oxidation of the P = S to P = O, the second is hydrolysis owing to phosphatases attacking P—S—C and carboxyesterases attacking $-COOC_2H_5$ linkages (see Fig. 3). The initially formed products may suffer further degradation leading to a variety of hydrolytic products; the ultimate product being inorganic phosphate or thiophosphate.

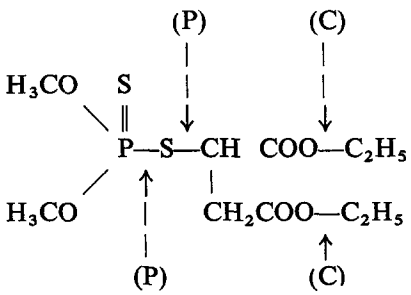


FIG. 3. Arrows indicate hydrolysis by phosphatases (P), or carboxyesterases (C).

Malaoxon (II), represents the major metabolite. Its percentage reaches about 25 per cent of the total metabolites and is probably produced mainly by an enzymatic oxidation of Malathion. Such oxidation of the P=S to P=O bond is known to occur in the larva of cotton leaf worm,[22] and other insects, e.g. American cockroach, house-fly[23] and boll-worm larva.[24]

Of the water soluble acidic substances, eight metabolites have been identified (Table 1).

A major site of enzymatic attack on Malathion seems to occur at the P—O-alkyl ester bond, followed by hydrolysis of the P—S bond, leading to the formation of thiophosphoric acid. It constitutes a major acidic metabolite and contributes to about 24 per cent of the total hydrolytic components.

Hydrolysis at the ester linkage(s) is influenced by the action of carboxyesterases on Malathion and leads to the formation of the mono- (IV) and diacid derivatives (V). The results suggest that the phosphatase action is the major hydrolytic mechanism.

*O,O*-Dimethylphosphorodithioic acid (III) could be isolated in relatively small percentage from the anion exchanger. It could be produced by a phosphatase attack of Malathion or the mono- and diacid derivatives at the S—C linkage.

The formation of *O,O*-dimethylphosphorothioic acid (VI) may be attributed either to enzymic oxidation of dithioate (III) or to hydrolysis of P—S bond of Malathion or the S—C bond of Malaoxon. Dimethyl phosphate (VIII) is probably produced by oxidation of VI and/or III or by cleavage of the P—S bond of Malaoxon (II).

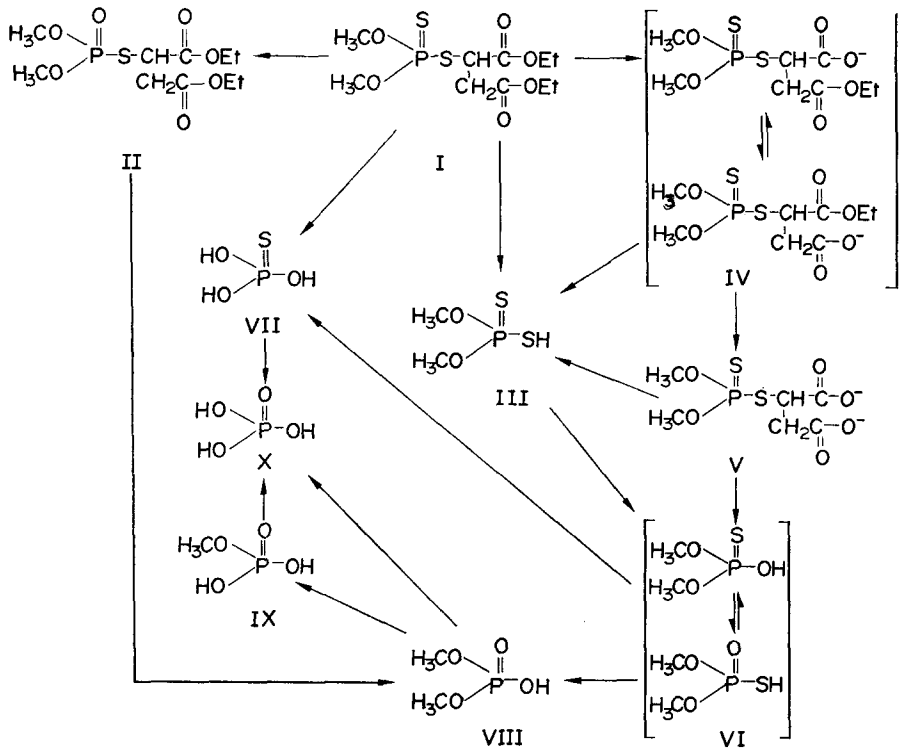


FIG. 4. Suggested scheme for the metabolic pathway of Malathion in *Spodoptera* larva.

The formation of monomethyl phosphate (IX) and/or phosphoric acid (X) may represent secondary cleavage of one or more of the original hydrolytic metabolites.

Neither des-methylated nor monodemethylated Malathion could be identified in the present investigation.

In the rat, the urine contains negligible amount of unchanged Malathion and about 1 per cent of the injected dose as Malaoxon (II). The low percentage of II may be due to the low oxidation of Malathion to P=O analogue in rat or due to fast degradation of the formed Malaoxon.

Many of the Malathion metabolites obtained from *Spodoptera* larva and rat were similar to those previously reported for insects[2–8] and mammals.[2,9,10]

Figure 4 illustrates the possible sites of metabolic attack of Malathion.

## REFERENCES

1. J. B. BOURKE, E. J. BRODERICK, L. R. HACKLER and P. C. LIPPOLD, *J. Agr. Food Chem.* **16,** 585 (1968).
2. H. R. KRUEGER and R. D. O'BRIEN, *J. Econ. Entomol.* **52,** 1063 (1959).
3. W. S. BIGLEY and F. W. PLAPP, JR., *J. Insect Physiol.* **8,** 545 (1962).
4. W. C. DAUTERMAN and F. MATSUMURA, *Science* **138,** 694 (1962).
5. F. MATSUMURA and A. W. A. BROWN, *J. Econ. Entomol.* **54,** 1176 (1961).
6. F. MATSUMURA and W. C. DAUTERMAN, *Nature* **202,** 1356 (1964).
7. F. MATSUMURA and G. VOSS, *J. Econ. Entomol.* **57,** 911 (1964).
8. F. MATSUMURA and G. VOSS, *J. Insect Physiol.* **11,** 147 (1965).
9. R. B. MARCH, T. R. FUKUTO, R. L. METCALF and M. G. MAXON, *J. Econ Entomol.* **49,** 185 (1956).
10. F. W. SEUME and R. D. O'BRIEN, *J. Agr. Food Chem.* **8,** 36 (1960).
11. F. MATSUMURA and G. M. BOUSH, *Science* **153,** 1278 (1966).
12. D. I. DARROW and F. W. PLAPP, JR., *J. Econ. Entomol.* **53,** 777 (1960).
13. R. D. O'BRIEN, *J. Econ. Entomol.* **50,** 159 (1957).
14. P. KOIVISTOINEN, A. KARINPÄÄ and M. KÖNÖNEN, *J. Agr. Food Chem.* **12,** 555 (1964).
15. P. KOIVISTOINEN, L. VANHANEN and E. M. KOSKINEN, *J. Agr. Food Chem.* **13,** 334 (1965).
16. F. MATSUMURA, *J. Econ. Entomol.* **53,** 452 (1960).
17. J. E. CASIDA, *Acta Chem. Scand.* **12,** 1691 (1958).
18. P. R. CHEN, W. P. TUCKER and W. C. DAUTERMAN, *J. Agr. Food Chem.* **17,** 86 (1969).
19. J. E. CASIDA, P. K. CHAPMAN and T. C. ALLEN, *J. Econ. Entomol.* **45,** 568 (1952).
20. C. S. HANES and F. A. ISHERWOOD, *Nature* **164,** 1107 (1949).
21. F. W. PLAPP and J. E. CASIDA, *Analyt. Chem.* **30,** 1622 (1958).
22. S. M. A. D. ZAYED, A. HASSAN and I. M. I. FAKHR, *Biochem. Pharmac.* **17,** 1339 (1968).
23. U. E. BRADY, JR. and B. W. ARTHUR, *J. Econ. Entomol.* **56,** 477 (1963).
24. D. L. BULL, D. A. LINDQUIST and J. HACSKAYLO, *J. Econ. Entomol.* **56,** 129 (1963).