Biochemical Pharmacology, 1965, Vol. 14, pp. 1577–1584. Pergamon Press Ltd., Printed in Great Britain.

METABOLISM OF ORGANOPHOSPHORUS INSECTICIDES—V.
MECHANISM OF DETOXIFICATION OF DIPTEREX* IN *PRODENIA LITURA* F.

A. HASSAN, S. M. A. D. ZAYED and F. M. ABDEL-HAMID

Dept. of Biology, Atomic Energy Establishment, Cairo, and the National Research Centre, Dokki, Cairo.

(*Received* 29 *December* 1964; *in revised form* 6 *March* 1965; *accepted* 6 *April* 1965)

Abstract—The metabolism of Dipterex in the cotton leaf worm (*Prodenia litura*) has been investigated *in vivo*, using ^{32}P- and ^{14}C-labelled insecticide. From the topically applied dose (2 mg/g insect), 40–45 per cent was excreted as ^{32}P-labelled hydrolytic products during 20 hr. The first major ^{32}P-labelled metabolite is produced by hydrolysis of both *O*-methyl ester linkages, and eliminated as glucuronide. This metabolite constitutes 65–75 per cent of the total metabolites output. The second metabolite contributes to 25–30 per cent, and proved to be dimethylphosphate. Monomethylphosphate (about 5 per cent) was also identified as a metabolic product. Radioactive carbon dioxide in the expired air accounted partly for the fate of the methyl groups of the insecticide. The possible metabolic pathways have been discussed.

INTRODUCTION

IN THE last few years, the general belief that Dipterex is effective against *Prodenia litura*[1] has led to a series of studies involving the larva[2, 3] and the cotton plant.[4] The low toxicity of the insecticide to the larvae has been ascribed—at least partly—to the absence of cholinesterase.[2] However, a contributing factor may well be a high rate of detoxification of Dipterex. For this reason, a detailed study of the metabolites, and rate of detoxification has been carried out *in vivo* using ^{32}P- and ^{14}C-labelled Dipterex.

MATERIAL AND METHODS

^{32}P-labelled Dipterex has been prepared according to the procedure described by Hassan and Zayed.[5] This ^{32}P-labelled compound possessed a specific activity of 0·6 mc/g. The same method has been adopted for the preparation of ^{14}C-labelled. Dipterex in which the two methyl groups are ^{14}C-labelled. This substance had a specific activity of 0·5 mc/g.

In this investigation, laboratory reared larvae of *Prodenia litura* F. (5–6 instars) have been used. A certain dose of the radioactive insecticide dissolved in acetone (2 mg/g per insect; about 200,000 cpm) was applied topically. After 20 hr, the collected excreta were extracted with distilled water. The water was next extracted several times with chloroform which removes the major part of the unchanged insecticide. The water layer was then concentrated under vacuum.

* 0,0-dimethyl-2,2,2-trichloro-1-hydroxyethyl phosphonate.

Experiments with ^{32}P-*labelled Dipterex*

The water concentrate was applied on an anion exchanger column, 15 × 1 cm (Dowex 1-x8, Cl^-, 100–200 mesh), and washed with distilled water till no ^{32}P-activity was detectable in the eluate. This is to ensure removal of the last traces of the unchanged compound. Elution of the hydrolytic products proceeded as described by Zayed and Hassan,[3] using hydrochloric acid solutions of different concentrations. Radioactive samples eluted at different pHs were analysed by paper chromatography.

Experiments with ^{14}C-*labelled Dipterex*

A part of the water concentrate was used for the detection of ^{14}C-formate as described in a previous paper.[5] Another part was analysed for other possible metabolites by radio-paper chromatography. For the collection of expired air, metabolic cages have been used.[6] The $^{14}CO_2$ was trapped by 1N sodium hydroxide solution and determined as $Ba^{14}CO_3$.

Radio-measurements

^{32}P-activity eluted from the anion exchanger was determined in solution (3ml), and all measurements were carried out in an end window counter. All data were corrected for decay and background. No allowance has been made for self-absorption.

^{14}C-activity in the water and chloroform layers was determined according to the procedure of Aronoff,[7] using VanSlyke-Folch reagent.[8] All ^{14}C-activity measurements (determined as $Ba^{14}CO_3$) were corrected for self-absorption and background.

The radiometric assay of the paper chromatograms was carried out using a manually operated devise with a GM tube connected to a scaler. The radioassay of the chromatograms provided only ratios of the different metabolites, and needed to be corrected only for background.

Enzyme activity determinations

The catalatic activity of catalase in the larval hemolymph has been determined according to the method described by Euler and Josephson;[9] using sodium thiosulphate for titrating the residual hydrogen peroxide. The enzyme was also assayed by the method devised by Miller,[10] who used the destructive effect of the substrate for determining the catalase activity. The peroxidatic action of the enzyme has been tested, using ^{14}C-sodium formate as an H-donor.[11]

The presence of aldehyde oxidase in the larval hemolymph has been investigated by the method of Gordon *et al.*;[12] using the Warburg constant volume respirometer.

For sake of comparison, mouse liver homogenates have been assayed for catalase and aldehyde oxidase activities, under similar conditions.

RESULTS

^{32}P-*labelled metabolites*

Figure 1 illustrates an elution curve obtained by eluting the acidic ^{32}P-labelled metabolites, with different hydrochloric acid concentrations. It was found that about $\frac{1}{3}$ of the radioactivity could be eluted at pH 1·7. The major part of activity—constituting the second peak—was elutable at pH 0·5. With further lowering of pH to 0, no more radioactivity could be detected in the eluate. In another experiment, it was shown that the metabolite(s) of the second peak were readily eluted at pH 0·9; though not as rapidly and completely as when eluted at pH 0·5.

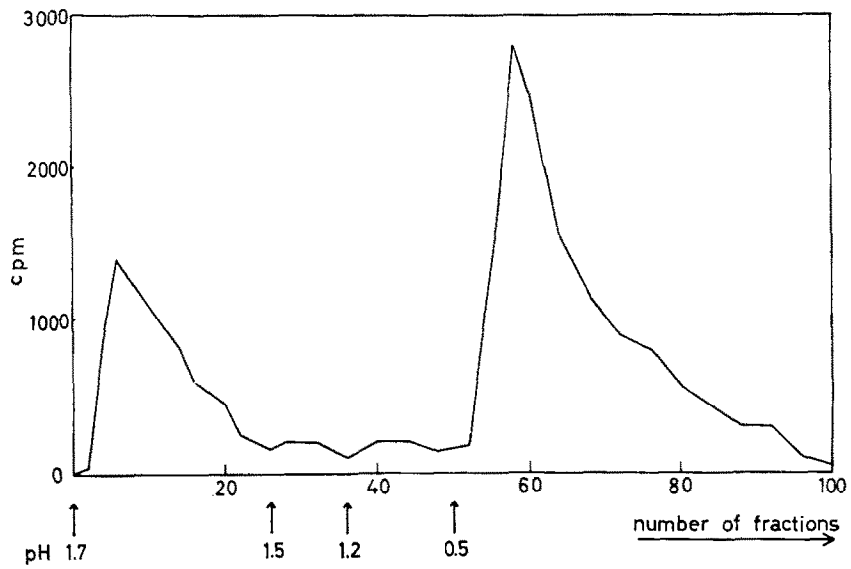

FIG. 1. Acidic ^{32}P-labelled products recovered from the anion exchanger with hydrochloric acid of pH 1·7, 1·5, 1·2, and 0·5. Each fraction contains 3 ml eluent.

In one experiment, the acidic metabolite(s) belonging to the first peak in Fig. 1 were eluted completely at pH 1·5; till no more radioactivity was detectable in the eluate. The ^{32}P-activity of the second peak was collected at pH 0·5 in the same way. All the acidic metabolites accounted for 44·2 per cent of the initially applied dose (Table 1). The rest of the dose is presumably lost in the surroundings during the movement of the larvae; since the latter contained only 1–2 per cent of the applied radioactivity after 20 hr. The chloroform extract proved to contain only ^{32}P-labelled Dipterex; as tested by radio-paperchromatography (R_f 0·95 in n-butanol-pyridine-water 12 : 8 : 6).[13]

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

^{32}P-activity	cpm	Percentage recovery (applied dose = 100%) = 1180,000 cpm	Metabolites identified in the eluate	*Percentage metabolite
eluted at pH 1·5	163,900	13·9	monomethyl-phosphate dimethyl phosphate	5 27
eluted at pH 0·5	358,000	30·3	coupled metabolite†	68
total	521,900	44·2		100

* Percentages of metabolites are estimated—relative to each other—from paper chromatographic studies (100% = total hydrolytic products).

† No other acidic substance (labelled or non-labelled) was detected in this fraction as tested by paper chromatography, where the chromatogram was sprayed with methyl orange indicator.

Radio-paperchromatography of the acidic metabolites (hydrolytic products) in 2-propanol-ammonium hydroxide-water 75 : 24 : 1[14] revealed the presence of three ^{32}P-labelled compounds (Fig. 2). Two metabolites; identified as mono- and dimethyl phosphates, were completely elutable at pH 1·5. The third metabolite—excreted in a coupled form—was the only ^{32}P-labelled substance eluted at pH 0·5 (Fig. 2). The relative ratios of the three metabolites are indicated in Table 1. For characterization of metabolites, the original water extract—after removing the unchanged insecticide—was paperchromatographed in four different solvent systems. The R_f values of the three metabolites in these systems are shown in Table 2.

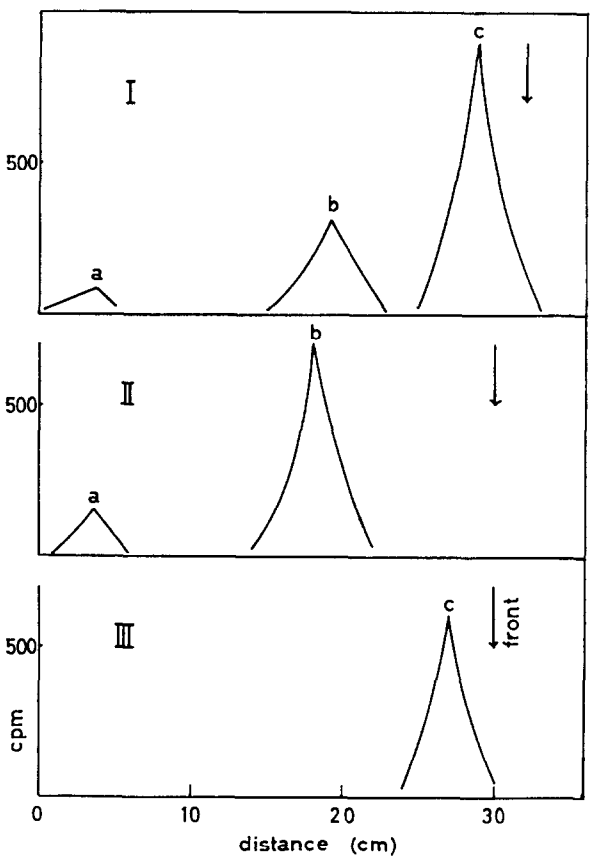

FIG. 2. Radio-paperchromatography in 2-propanol-ammonium hydroxide-water (75 : 24 : 1) of:
I, Original water extract, after removing Dipterex.
II, Substances eluted from the anion exchanger at pH 1·5
III, Substances eluted from the anion exchanger at pH 0·5.
a. monomethylphosphate; b. dimethylphosphate; c. coupled metabolite in the glucuronide form. All substances are ^{32}P-labelled.

^{14}C-*labelled metabolites*

The total ^{14}C-labelled compounds—recovered from the excreta and the expired air—accounted for 35–45 per cent of the applied dose. 10–14 per cent of the ^{14}C-labelled metabolites was eliminated as $^{14}CO_2$ in the expired air within 20 hr. This

gives an average value of 4·7 μmole/hr/100 g insect, for the rate of hydrolysis of Dipterex at the O-methyl ester bond(s).

In addition to the unchanged insecticide, the chloroform extract proved to contain a ^{14}C-labelled compound of unknown nature. It possesses R_f 0·76 in system D (cf. Table 2). It has been estimated to account for 40–60 per cent of the total ^{14}C-labelled metabolites.

TABLE 2. R_f VALUES OF THE DEGRADATION PRODUCTS OF P^{32}-LABELLED DIPTEREX IN FOUR DIFFERENT CHROMATOGRAPHIC SYSTEMS

System*	R_f		
	monomethyl-phosphate	dimethyl phosphate	coupled metabolite
A 2-propanol-ammonium hydroxide-water (75 : 24 : 1)	0·12	0·60	0·90
B n-butanol saturated with 1·5N ammonium hydroxide	—	0·08	0·61
C n-butanol saturated with ammonium hydroxide	0·08	0·25	0·73
D n-butanol-pyridine-water (12 : 8 : 6)	0·05	0·17	0·75

* Development time for systems A and D was 15 hr and for B and C 10 hr.

Radio-paperchromatography of the aqueous layer revealed the presence of only two ^{14}C-labelled substances, accounting for 30–40 per cent of the total ^{14}C-labelled metabolites. In system A (Table 2), two spots corresponding to R_f 0·12 and 0·60 (mono- and dimethylphosphates respectively) have been obtained; in about the same ratio as found with ^{32}P-labelled metabolites. However, a third ^{14}C-labelled metabolite could not be traced in the aqueous layer.

The ^{14}C-activity remaining in the larvae after 20 hr was found not to exceed 4 per cent of the applied dose.

Structure of the coupled metabolite

The eluate collected at pH 0·5, and which contains only one ^{32}P-labelled substance, was tested for the presence of the trichloro portion of the Dipterex molecule by means of modified Fujiwara test.[15] After dichromate oxidation, a crimson colour has been formed; indicating that the phosphonate bond of that metabolite is still intact.

A part of the eluate at pH 0·5 was rendered alkaline, boiled for 10 min, and the products were investigated by paper chromatography. In system A (Table 2), the substance possessing R_f 0·90 disappeared, and instead, the ^{32}P-activity remained on the base line. This product gives a yellow colour immediately after spraying the chromatogram with Bandurski reagent;[16] suggesting the presence of orthophosphate. Evidence for the formation of orthophosphate has been directly obtained by chromatographing the boiled alkaline solution in methyl alcohol–2N ammonium hydroxide (7 : 3), and in ethyl acetate–formamide–pyridine (1 : 2 : 1). The radioactivity possessed R_f 0·31 and 0·50 in the first and second systems respectively.[17]

The boiled alkaline solution was acidified and extracted with ether. The dried ether extract (over anhydrous magnesium sulphate) gave positive Molisch's test, and blackened ammoniacal silver nitrate solution. The ether extract was then paperchromatographed in n-butanol-acetic acid-water (4 : 1 : 5),[18] and in pyridine–ethyl acetate–acetic acid–water (5 : 5 : 1 : 3).[19] The spots were made visible by spraying with

ammoniacal silver nitrate solution, or with methyl orange indicator in alcohol. Only one substance has been detected with R_f 0·12 and 0·30 in the first and second systems respectively. Glucuronic acid (as a reference substance) gave similar R_f values in these systems.

Enzymatic studies

The activity of the catalase-like substance in the hemolymph of *Prodenia litura* has been investigated by determining the reaction constant (k) according to the equation: $k = 1/t \log x_0/x$; where t = time in minutes, x_0 = vol. of titrant (ml) at zero time, x = vol. of titrant (ml) after elapse of time t. The assay of eight samples gave values 100–150 times less active than the enzyme of the mouse liver. The values obtained by Miller's method were also within this range. In a qualitative manner, this catalase-like substance proved to be capable of oxidizing ^{14}C-formate peroxidatically.

A substance capable of oxidizing aldehydes (aldehyde oxidase-like substance) was found in the larval hemolymph. It showed an activity 6–10 times less potent than the aldehyde oxidase of the mouse liver (compared values were the result of five determinations).

DISCUSSION

The metabolism of Dipterex in *Prodenia litura* has been recently investigated *in vitro* using ^{32}P-labelled insecticide.[3] Monodemethylated Dipterex and monomethylphosphate could be identified as degradation products. Though the compound is fairly rapidly absorped by the larvae, high doses proved to be non-fatal to the insect, and the absence of cholinesterase is supposed to account for the low toxicity of Dipterex.[2]

In this investigation, an approach has been attempted to account for the low toxicity of the insecticide in terms of high detoxification rates. As a matter of fact, the excretion of 800–900 μg ^{32}P-labelled metabolites per gram insect, during 20 hr, clearly illustrates that the compound is detoxified at an exceptionally high rate. This may also provide the answer for the low toxicity of the insecticide to the larvae.

It is believed that the hemolymph and the gut of the larva are the chief participants responsible for the degradation of the insecticide.[3] From the data reported in this paper, it may be concluded that the mechanism of detoxification of Dipterex involves mainly the hydrolysis of the O-methyl ester linkage(s), and to a smaller extent the phosphonate bond. Figure 3 illustrates a scheme for the possible metabolic pathways of Dipterex *in vivo*.

The radioactive CO_2 eliminated in the expired air could account for only a small percentage (10–14 per cent) of the total ^{14}C-labelled metabolites. The enzymatic studies (catalase and aldehyde oxidase) carried out in this investigation were aimed to explain the elimination of $^{14}CO_2$; in much the same manner as in the rat.[5] As a matter of fact, the presence of both enzymes in the larval hemolymph suggests the pathway indicated in Fig. 3; catalase being involved in the oxidation of methanol to formaldehyde and formate to CO_2. In this connection, it is worth mentioning that ^{14}C-formate could not be detected in the excreta; contrary to what is known to occur in mammals.[5, 20] The formation of the coupled ^{32}P-labelled metabolite, with no methyl groups of the original compound (Fig. 3), in quantities ranging from 65–75 per cent;

strongly suggests that there must be some metabolite(s)—other than $^{14}CO_2$—into which is incorporated the ^{14}C-atoms of the methyl groups. In fact, such a compound was found in the chloroform layer. It does not contain phosphorus, and is most probably devoid of an acidic group.

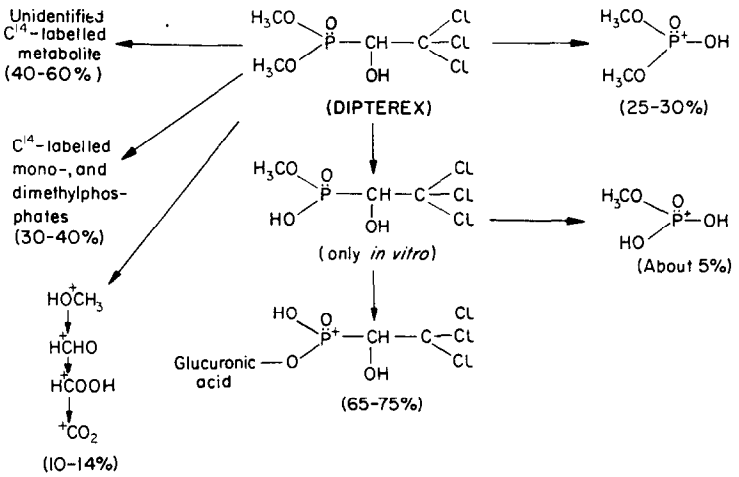

FIG. 3. Possible metabolic pathways of Dipterex in *Prodenia litura* + *Radioactive atom.* Percentages are related to either ^{32}P- or ^{14}C-labelled total metabolites output.

The formation of three ^{32}P-active metabolites indicates that both hydrolytic processes (taking place at P-OCH_3 and C-P bonds) proceed independently (Fig. 3). The P-OCH_3 cleavage proceeds at a higher rate than that of the esterase (C-P hydrolysis). The production of monomethylphosphate supports the formation of monodemethylated Dipterex *in vitro*;[3,21] being its immediate precursor. Though dimethylphosphate is excreted quantitatively unchanged in the rat,[22] the possibility that it may serve as a precursor for monomethylphosphate cannot be outruled. Monodemethylated Dipterex is probably also a precursor for the coupled metabolite. The immediate precursor of the latter—2,2,2-trichloro-1-hydroxyethyl phosphonic acid—is believed to be formed in mammalian nervous tissue,[21]

The structure suggested for the coupled metabolite lends support from several observations, some of which have been indicated. It seems that the dihydroxy compound (2,2,2-trichloro-1-hydroxyethyl phosphonic acid) does not possess enough acidity to be excreted as such; and has to be coupled for elimination (contrary to mono- and dimethylphosphates). The coupling of this product with a normal metabolic compound (in this case glucuronic acid) constitutes a primary defense mechanism. The linkage between 2,2,2-trichloro-1-hydroxyethyl phosphonic acid and glucuronic acid is probably of the glucoside type which can be split by acid hydrolysis (and also to a smaller extent by alkali). This has been concluded by an observation that the coupled metabolite does not reduce ammoniacal silver nitrate solution, except after being hydrolysed; thus freeing the reducing group of glucuronic acid. In this case, it is believed that the union is through the hydroxyl group on carbon 1 of the lactone structure.

Glucuronic acid is believed to be coupled with only one of the free OH groups of the phosphonic acid. This has been concluded by considering two parameters. First, one OH group should remain free to contribute to the acidity of the metabolite. Second; the metabolite gives a blue colour with Bandurski reagent,[16] while Dipterex does not give the colour; indicating that at least one valency of the three valencies occupied with organic groups should not be organic-bound, if the blue colour is to be produced.

REFERENCES

1. A. A. M. KAMEL, A. SHOEB, A. HANNA and S. SOLIMAN. *Agr. Res. Rev. (Cairo)*, **36**, 1 (1958); C. A. **53**, 12571 (1959).
2. A. HASSAN and S. M. A. D. ZAYED. *Naturwissen*, **52**, 18 (1965).
3. S. M. A. D. ZAYED and A. HASSAN. *Canad. J. Biochem.*, in press (1965).
4. I. Y. MOSTAFA, A. HASSAN and S. M. D. A. ZAYED. *Z. Naturforsch.*, **20b**, 67 (1965).
5. A. HASSAN and S. M. A. D. ZAYED. *Canad. J. Biochem.*, in press (1965).
6. R. W. COWGILL and A. B. PARDEE. *Experiments in Biochemical Research Techniques*, pp. 155, N.Y., John Wiley, Inc. (1957).
7. S. ARONOFF. Techniques of Radiobiochemistry, pp. 45, The Iowa State College Press (1957).
8. D. D. VAN SLYKE and J. FOLCH. *J. biol. Chem.*, **136**, 509 (1940).
9. H. VON EULER and K. JOSEPHSON. *Liebigs Ann.*, **455**, 1 (1927).
10. H. MILLER. *Biochem. J.*, **68**, 275 (1958).
11. H. AEBI and A. HASSAN. *Helv. chim. Acta*, **43**, 544 (1960).
12. A. H. GORDON, D. E. GREEN and V. SUBRAHMANYAN. *Biochem. J.*, **34**, 764 (1940).
13. F. ACREE Jr., F. H. BABERS and N. MITLIN. *J. Econ. Entom.* **49**, 808 (1956).
14. F. W. PLAPP and J. E. CASIDA. *Analyt. Chem.*, **30**, 1622 (1958).
15. P. A. GIANG, W. P. BARTHEL and S. A. HALL. *J. Agr. Food Chem.*, **2**, 1281 (1954).
16. R. S. BANDURSKI and B. AXELROD. *J. biol. Chem.*, **193**, 405 (1951).
17. S. ARONOFF. Techniques of Radiobiochemistry, pp. 194, The Iowa State College Press (1957).
18. S. M. PARTRIDGE. *Biochem. J.*, **42**, 238 (1948).
19. F. G. FISCHER and H. DORFEL. *Z. Physiol. Chem.*, **302**, 10 (1955).
20. H. AEBI, H. KOBLET and J. P. VON WARTBURG. *Helv. Physiol. Acta*, **15**, 384 (1957).
21. A. HASSAN, S. M. A. D. ZAYED and F. M. ABDEL-HAMID. *Canad. J. Biochem., in press* (1965).
22. B. W. ARTHUR and J. E. CASIDA. *J. Agr. Food Chem.*, **6**, 360 (1958).