The Oxidative Dealkylation of Insecticidal Phosphoric Acid Triesters by Mammalian Liver Enzymes

By C. DONNINGER, D. H. HUTSON and B. A. PICKERING
Shell Research Limited, Tunstall Laboratory, Sittingbourne, Kent, U.K.

(Received 27 September 1971)

1. The dealkylation of the insecticidal phosphoric acid triester, 2-chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate, proceeds in mammalian liver slices via an oxidative mechanism and not by hydrolysis. 2. The enzyme that catalyses the reaction is located in the microsomal fraction of liver homogenate and is dependent for activity on molecular oxygen and NADPH. 3. There are large species differences between rat, mouse, rabbit and dog in the activity of the enzymes, the relative rates of dealkylation being 1, 8, 24 and 88 respectively in liver slices. 4. Dimethyl and di-isopropyl phosphate triesters are also dealkylated by rabbit liver microsomal preparations. 5. The mechanism of dealkylation involves hydroxylation at the α -carbon atom of an alkyl group, which is removed as the corresponding aldehyde, and is thus analogous to that of similar reactions catalysed by the microsomal mixed-function oxidases. 6. The relevance of these findings in the toxicology of phosphoric acid triesters is discussed.

The detoxification of an insecticidal phosphoric acid triester in mammals may be effected by the removal of one substituent from the molecule to form a phosphate diester, a class of compound which in general possesses no anticholinesterase properties (Aharoni & O'Brien, 1968). Many phosphoric acid triesters are metabolized by both routes (*a*) and (*b*) which are illustrated in Scheme 1 (Plapp & Casida, 1958) and the mechanism of reaction (*a*) has received some attention (Aldridge, 1953; Mounter, 1954; Augustinsson, 1958). Two studies of the metabolism in dogs and rats of the vinyl phosphates, chlorfenvinphos [2-chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate] (Hutson *et al.*, 1967) and tetrachlorvinphos [2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate] (Akintonwa & Hutson, 1967), revealed that the initial detoxification reaction could follow one or both of these routes. The detoxification of chlorfenvinphos by route (*b*) accounts in part for the resistance of the dog to the action of large doses of the insecticide (Hutson & Hathway, 1967). Similarly, the demethylation of Sumithion [*OO*-dimethyl-*O*-(3-methyl-4-nitrophenyl)phosphorothioate] is thought to be important in its relative non-toxicity to mammals (Hollingworth *et al.*, 1967). In a molecule where OX is the favoured leaving group (see Scheme 1), it is difficult to conceive of an esterase-type mechanism for reaction (*b*) which would involve ethoxide ion as the leaving group. We therefore considered that the mechanism of reaction (*b*) should be investigated. The present paper deals with the location of an enzyme that catalyses the de-ethylation of chlorfenvinphos in mammals and a more detailed study of the reaction by using enzymes from rabbit liver.

Experimental

Materials

2-Chloro-1-(2,4-dichlorophenyl)[^{14}C]vinyl diethyl phosphate (vinyl-labelled chlorfenvinphos). We are indebted to Shell Development Co., Modesto, Calif., U.S.A., for synthesizing this material. It was purified before use as described by Hutson *et al.* (1967).

2-Chloro-1-(2,4-dichlorophenyl)vinyl [^{14}C]ethyl phosphate (ethyl-labelled chlorfenvinphos). 2-Chloro-1-(2,4-dichlorophenyl)vinyl ethyl hydrogen phosphate (2.6g) was dissolved in dry ether (40ml) and dicyclohexylcarbodi-imide (0.7g) in dry ether (10ml) was added. After 15 min, the solution was evaporated to 25 ml, crystals of dicyclohexylurea were removed, and di-[2-chloro-1-(2,4-dichlorophenyl)vinyl] diethyl pyrophosphate was allowed to crystallize overnight at 2°C to yield 1.7g (65%) of colourless crystals, m.p. 97°C. (Found: C, 37.3; H, 2.7; Cl, 32.7; P, 9.6; $\text{C}_{20}\text{H}_{18}\text{O}_7\text{Cl}_6\text{P}_2$ requires: C, 37.2; H, 2.8; Cl, 33.0; P, 9.6). The compound was stable when stored dry under vacuum for 4 days at room temperature but deteriorated slowly after this time. The pyrophosphate (54mg) was dissolved in 0.2 ml of dry acetone, and 10 μl of ethanol was added. This mixture was introduced into a cooled (–80°C) ampoule containing 100 μCi of [^{14}C]ethanol (10.5 mCi/mmol) (The Radiochemical Centre, Amersham, Bucks., U.K.) and allowed to warm to room temperature. A piece of clean sodium (2mg) was introduced and the mixture left for 30 min at room temperature. The contents of the tube were then purified on two 20 cm $\times$ 20 cm $\times$ 1 mm t.l.c. plates, developed in benzene–methanol

Scheme 1. Primary routes of detoxification of insecticidal phosphoric acid triesters

R = alkyl; X = leaving group (e.g. *p*-nitrophenyl).

(25:1, v/v), and the chlorfenvinphos zone (R_F 0.4; 92% of radioactivity on the plate) was eluted with acetone. Radioassay by scintillation counting indicated a total yield of ^{14}C of 8.18 μCi . Part of the stock solution was diluted 1250-fold to a suitable concentration for analysis by g.l.c. The column consisted of Celite 100–120 coated with 2% Oronite and 0.2% Epikote 1001; the temperature was 190°C; the gas was O_2 -free N_2 at 140 kN/m² (20 lb/in²). The ^{14}C -labelled-material was compared with a standard sample of technical chlorfenvinphos of 92% purity (β -form). The α -isomer was clearly distinguishable in the technical sample but was absent from the synthetic ^{14}C -labelled sample. The concentration of β -chlorfenvinphos in the stock solution was 57.27 $\mu\text{g}/\text{ml}$. Thus the specific radioactivity of the product was 511.3 $\mu\text{Ci}/\text{mmol}$ and yield (based on ^{14}C) was 8.2%.

2-Chloro-1-(2,4,5-trichlorophenyl)[^{14}C]vinyl dimethyl phosphate. This material was obtained from Shell Development Co., Modesto, Calif., U.S.A.

2-Chloro-1-(2,4-dichlorophenyl)vinyl ethyl hydrogen phosphate. This compound was synthesized as described by Hutson *et al.* (1967).

Dialkyl aryl phosphates: general method. 1-Naphthol (0.125 mol) was dissolved in a solution of 0.13 mol of NaOH in 30 ml of water. Dialkyl phosphoryl chloride (0.1 mol) was added and the reaction mixture shaken for 1 h. The mixture was then diluted with 500 ml of 2% (w/v) NaOH and the product extracted with 500 ml of chloroform. The extract was washed with 2% NaOH until the washings were free of 1-naphthol. Finally the solution was washed with water, dried with anhydrous Na_2SO_4 and evaporated to a syrup. Yields were 60–70%. The products were shown to be pure by t.l.c. in chloroform on Merck silica-gel plates. The structure of each product was confirmed by n.m.r. spectroscopy. The following were prepared in this way: dimethyl 1-naphthyl phosphate, diethyl

1-naphthyl phosphate and di-isopropyl 1-naphthyl phosphate.

Dialkyl 1-[^{14}C]naphthyl phosphate. The radioactive compounds were prepared as described above, by scaling down the reaction to a 1.0 or 0.5 mmol level. 1-[^{14}C]Naphthol was purchased from The Radiochemical Centre and diluted with non-radioactive carrier material such that specific radioactivity was 71.4 $\mu\text{Ci}/\text{mmol}$. Products were purified by t.l.c. on Merck preparative (2 mm) plates of silica gel F254, with benzene-methanol (9:1, v/v) as solvent.

Cofactors and other chemicals. Nicotinamide and glucose 6-phosphate were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; NAD^+ , NADH, NADP^+ and NADPH from Sigma (London) Chemical Co., London S.W.6, U.K.; and glucose 6-phosphate dehydrogenase from C. F. Boehringer und Soehne G.m.b.H, Mannheim, W. Germany. *p*-Nitroanisole and aminopyrine were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., chlorpromazine hydrochloride and hexobarbital from May and Baker Ltd., Dagenham, Essex, U.K. Sesamex and triethyl phosphate were supplied by the Woodstock Agricultural Research Centre, Sittingbourne, Kent.

Animals

Rats and mice. Male CFE rats and male CF1 mice (both maintained as specific-pathogen-free colonies in this laboratory) were used as sources of liver.

Rabbits. Male New Zealand Whites were used for sources of liver for the preparation of homogenates and microsomal fractions.

Dog. A healthy adult beagle hound was used as a source of dog liver.

Methods

Preparation of liver tissue and incubation with chlorfenvinphos. Livers were taken from animals killed by a blow (except for the dog, which was exsanguinated under barbiturate anaesthesia). All operations were then carried out at 2°C in a modified Krebs-Ringer buffer of the following composition: 0.9% NaCl (100 parts); 1.15% KCl (4 parts); 1.22% CaCl_2 (3 parts); 3.82% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1 part); 0.63% NaHCO_3 (1 part); 4.5% (w/v) glucose (1 part); and 0.1 M-potassium phosphate buffer, pH 7.4 (20 parts). The livers were washed in the buffer, sliced finely and samples (1 g wet wt.) were added to identical flasks each containing 15 ml of the buffer and [^{14}C]chlorfenvinphos (evaporated from a hexane solution) at a final concentration of 0.2 mM. The unstoppered flasks were shaken for various times at 37°C and analysed, after homogenization in methanol by centrifugation, and analysis of the supernatant by paper chromatography. In the solvent used (butan-

1-ol-2M-NH₃, 1:1, v/v; top phase), the unchanged insecticide had R_F 0.9, and the product R_F 0.65, similar to that of 2-chloro-1-(2,4-dichlorophenyl)vinyl ethyl hydrogen phosphate (Hutson *et al.*, 1967). Radioactive zones on chromatograms were located by scanning with a Nuclear-Chicago Actigraph II equipped with a gas-flow cell utilizing a helium-butane (98.5:1.5, v/v) mixture. The extent of conversion of chlorfenvinphos into its metabolite was assessed from the relative areas under the radiochromatogram scan.

Subcellular location of enzyme activity in liver homogenates. Rabbit liver (76g wet wt.) was homogenized at 2°C in 0.1M-potassium phosphate buffer, pH7.4, containing 0.03M-nicotinamide. The whole homogenate was assayed by the method described above and found to contain a high activity of the enzyme. This was only so if the nicotinamide was included in the phosphate buffer. Enzyme activity in 0.25M-sucrose homogenates was less than the activity in phosphate-buffer homogenates. The homogenate was centrifuged successively at 700g (15 min), 8000g (20 min) and finally at 100000g (60 min). Assay of the various supernatant fractions indicated that the enzyme activity was present in the proportions 70:100:3 respectively, and therefore was associated largely with the microsomal fraction. This fraction was resuspended in the original homogenate volume of 0.03M-nicotinamide-0.1M-potassium phosphate buffer, centrifuged at 100000g for 60 min and resuspended. Such a preparation, referred to as washed microsomal fraction, usually contained 16-20mg of protein/g of liver.

Standard assay procedure. Cofactor requirements and other properties of the microsomal enzyme system were determined as far as possible by a standard assay procedure, as follows: 1ml of 0.4mM-[¹⁴C]chlorfenvinphos in hexane was evaporated in a 50ml Erlenmeyer flask. Microsomal suspension (2ml) and cofactor solution in 0.1M-phosphate buffer, pH7.4 (2ml), were added and the mixture was shaken, unstoppered, at 37°C. At appropriate times, 1ml portions of the mixture were transferred to 10ml of methanol. Precipitated protein was removed by centrifugation, and the supernatant was evaporated to dryness and partitioned between 10ml of hexane and 10ml of the phosphate buffer. After shaking for 1 min and centrifuging, the two layers were assayed for radioactivity by using Dobbs' (1963) scintillator and a Packard Tri-Carb liquid-scintillation spectrometer.

Measurement of protein. Protein content of microsomal fractions was measured by a quantitative modification of the biuret method described by Robinson & Hogden (1940).

Measurement of formaldehyde from the demethylation of tetrachlorvinphos. Formaldehyde was measured by the chromotropic acid method

(MacFadyen, 1945) by using 5 ml of reagent and 1 ml of test solution. Stock formaldehyde was standardized by the dimedone procedure (Yoe & Reid, 1941) and diluted to give solutions containing 0.5-6 µg/ml. Microsomal suspension (16mg of protein/ml, 4ml) was incubated with standard cofactor solution, but containing, in addition, 20mg of semicarbazide hydrochloride (4ml) and [¹⁴C]tetrachlorvinphos (final concentration 0.5mM). At 30 min and 1 h, the reaction mixture was sampled and analysed by the standard radioassay (percentage conversion 19 and 25% respectively). At the same time 3.5 ml portions were withdrawn and mixed with 3.5 ml of 20% (w/v) trichloroacetic acid solution, centrifuged and partially distilled (3 ml). Control experiments showed that such formaldehyde solutions (0.5-12.0 µg/ml) distilled unchanged in composition except for a 10% loss.

Results

Effect of liver slices on [¹⁴C]chlorfenvinphos

During incubation with slices of liver from various species, the substrate was converted into only one chromatographically identifiable product, 2-chloro-1-(2,4-dichlorophenyl)vinyl ethyl hydrogen phosphate. This product is the major metabolite of chlorfenvinphos *in vivo* in the dog and constitutes

Fig. 1. Relative rates of dealkylation of chlorfenvinphos in liver slices

[¹⁴C]Chlorfenvinphos was incubated at a concentration of 0.2 mM with liver slices from various species as described in the Experimental section. The degradation to 2-chloro-1-(2,4-dichlorophenyl)vinyl ethyl hydrogen phosphate was measured by paper radiochromatography in the butanol-NH₃ solvent described. ●, Rat; ▲, mouse; ■, rabbit; ○, dog.

about 30% of the metabolites formed by the rat (Hutson *et al.*, 1967). The rates of conversion of the substrate into the metabolite, expressed as percentage conversion versus time, are shown in Fig. 1. The relative initial rates of conversion are: rat 1; mouse 8; rabbit 24; dog 88. It is noteworthy that the values for the acute oral LD₅₀ values (mg/kg) of chlorfenvinphos in these species are: 10, 100, 500 and >12000 respectively. Dog kidney slices were also examined for the enzyme activity, but since they possessed only one-tenth of the activity of liver they were not studied further.

Location of the enzyme activity in liver fractions

In view of the rapid rate of metabolism of the insecticide by rabbit liver slices, this material was chosen as a source of the enzyme for further study. A homogenate of rabbit liver was separated by centrifugation into various supernatant fractions and these were examined for enzyme activity as described in the Experimental section. Most of the enzyme activity was associated with the microsomal fraction. Washed microsomal fraction, however, possessed activity only when soluble fraction was added to the incubation mixture. Thus cofactor requirements were demonstrated and experiments were carried out to determine these.

Properties of the enzyme

Washed microsomal fraction was prepared from rabbit liver in the presence of 0.03M-nicotinamide as described in the Experimental section. Enzyme

activity was measured by the standard procedure in the presence and absence of various cofactors. The results shown in Table 1 reveal that oxygen and NADPH (or an NADPH-generating system) are essential for maximal activity, and that NADH cannot efficiently replace NADPH. The enzyme activities quoted in Table 1 are based on initial rate measurements, this rate being constant provided that (i) microsomal protein concentration was below 1 mg/ml, (ii) time of incubation was less than 30 min and (iii) conversion of the substrate was less than 50%.

The effect of pH on the enzyme activity was determined by using standard incubation conditions but adding NADPH in place of the NADPH-generating system. Fig. 2 shows a sharp pH optimum for the reaction at pH 7.4. The effect of incubation temperature on enzyme activity was also examined, and Fig. 3 indicates the optimum activity at about 37°C. The rates of the reaction (R) at various substrate concentrations (S) were determined; the approximate K_m value was 14 μ M (95% fiducial limits 11–21 μ M).

Identification of the products of enzyme action

Chromatographic evidence had already suggested that the product of microsomal metabolism of chlorfenvinphos was 2-chloro-1-(2,4-dichlorophenyl)vinyl ethyl hydrogen phosphate. This was confirmed by isotope-dilution analysis. A reaction was carried out under standard conditions and the deproteinized product partitioned between hexane and buffer (pH 7.4). The radioactivity in the buffer phase was mixed with a known weight of authentic 2-chloro-1-(2,4-dichlorophenyl)vinyl ethyl hydrogen

Table 1. *Dependence of the rabbit liver microsomal enzyme activity on cofactors*

Mixed cofactor solution was composed of glucose 6-phosphate (10mM), glucose 6-phosphate dehydrogenase (1.0 unit/ml), MgCl₂ (5mM) and NADP⁺ (0.2 mM). NADPH and NADH were added as 2mM solutions. Mixtures of microsomal suspensions (2ml) (protein concn. 1.08 mg/ml) and cofactor solution (dissolved in 0.1 M-potassium phosphate buffer, pH 7.4) (2ml) were shaken with [¹⁴C]chlorfenvinphos (final concn. 0.1 mM) at 37°C and analysed by the radioassay procedure. When incubations were carried out anaerobically, the reaction flask, cooled to 0°C, was evacuated and the atmosphere replaced with N₂. This was repeated three times before warming up to 37°C.

Cofactor conditions	Rate of conversion (nmol of chlorfenvinphos/ min per mg of protein)
Mixed cofactors	1.47
Mixed cofactors minus NADP ⁺	0.23
Mixed cofactors minus glucose 6-phosphate dehydrogenase	0.12
Mixed cofactors minus glucose 6-phosphate	0.07
Mixed cofactors minus O ₂	0.14
NADPH plus O ₂	1.00
NADPH minus O ₂	0.08
NADPH plus O ₂ plus Mg ²⁺	1.00
NADH	0.23

Fig. 2. Effect of pH on the dealkylation of chlorfenvinphos by rabbit liver microsomal preparation

[^{14}C]Chlorfenvinphos (0.1 mM) was incubated with rabbit liver microsomal preparation. The conditions used were as described in Table 1 except that NADPH (5 mM) replaced the NADPH-generating system and pH variation was achieved by the use of different 0.1 M-potassium phosphate buffers.

Fig. 3. Effect of temperature on the dealkylation of chlorfenvinphos by rabbit liver microsomal fraction

The conditions used were as described for Fig. 2.

phosphate, which was then isolated and recrystallized three times. The method showed that at least 83% of the radioactivity in the aqueous phase was 2-chloro-1-(2,4-dichlorophenyl)vinyl ethyl hydrogen phosphate.

The fate of the ethyl group removed from the insecticide during metabolism by microsomal fraction was determined by using [^{14}C]ethyl-labelled chlorfenvinphos. A standard reaction mixture (8 ml) containing this substrate and twice-washed microsomal fraction was incubated in a sealed flask under pure O_2 for 1 h. The reaction was terminated by the addition of 20 ml of ethanol containing 200 mg of acetaldehyde. Part of this mixture (5 ml) was analysed by paper chromatography, which indicated that 86% of the chlorfenvinphos had been de-ethylated. The remainder of the mixture was then half distilled into a receiver at 0°C and the recovered acetaldehyde converted into its dimedone derivative, ethylidene bis(dimethyldihydroresorcinol), m.p. 140°C . This product had a specific radioactivity which indicated that 54% of the ethyl group removed from the substrate was recovered as acetaldehyde. The possibility that the ethyl group was liberated as ethanol and then partially oxidized to acetaldehyde was ruled out by the finding that [^{14}C]ethanol in a control reaction was not converted into [^{14}C]acetaldehyde. It was not possible to improve the recovery of [^{14}C]acetaldehyde by adding carrier before the microsomal enzyme reaction, because acetaldehyde inhibited the reaction.

Inhibition of chlorfenvinphos dealkylase

A series of standard reactions were prepared containing, in addition to chlorfenvinphos, a series of compounds known either to inhibit the microsomal mono-oxygenase(s) or to be metabolized by microsomal oxidation. Each compound was present in the same molar concentration as the substrate (i.e. 0.1 mM). Triethyl phosphate was also included in this series but at a concentration of 0.2 mM. After 10 min incubation, the percentage breakdown of chlorfenvinphos was measured in each case by the radioassay procedure, and these results are compared in Table 2.

Demethylation of a chlorfenvinphos analogue

2-Chloro-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate (tetrachlorvinphos) was found to be metabolized mainly by demethylation in the dog, in the same manner as chlorfenvinphos (Akintonwa & Hutson, 1967). When [^{14}C]vinyl-labelled compound was incubated with the microsomal preparation under standard conditions it was converted into a polar metabolite at almost twice the rate of conversion of chlorfenvinphos. The products of the reaction were examined by paper chromatography and the only detectable metabolite was 2-chloro-1-(2,4,5-trichlorophenyl)vinyl methyl hydrogen phosphate (R_f 0.7). The possibility of the liberation of the methyl group as formaldehyde was tested by the chromotropic acid method as described in the Experimental section. The yields of formaldehyde at 30 min and 1 h indicated

Table 2. *Effect of some compounds on the enzymic dealkylation of chlorfenvinphos*

Enzyme assays were carried out as described in the Experimental section. Sesamex is the 2-(2-ethoxyethoxy)ethyl 2,4-methylenedioxyphenyl acetal of acetaldehyde.

Compound	Concn. (mm)	Conversion of 0.1 mM-[¹⁴ C]- chlorfenvinphos in 10min (radioassay procedure) (%)
None (control reaction)	0.1	18.0
<i>p</i> -Nitroanisole	0.1	14.0
Amidopyrine	0.1	21.0
Hexobarbital	0.1	15.6
Chlorpromazine	0.1	16.0
Sesamex	0.1	12.2
Triethyl phosphate	0.2	18.0

conversions of 16 and 29% respectively. Percentage conversions measured by the radioassay procedure were 19% and 25%, indicating that the methyl group was removed as formaldehyde.

Dealkylation of dialkyl naphthyl phosphates

When diethyl [¹⁴C]naphthyl phosphate and diisopropyl [¹⁴C]naphthyl phosphate were incubated with rabbit liver microsomal preparation under the standard conditions, the initial rates of dealkylation were 0.4 and 0.37 nmol/min per mg of protein respectively. The products of the two reactions were shown by paper chromatography to be the corresponding monoalkyl naphthyl phosphates and not naphthol.

Discussion

The results in Table 1 show that the dealkylation reaction is dependent on NADPH and molecular oxygen, and therefore the enzyme system involved possesses the characteristics of a typical microsomal mixed-function oxidase (Mason, 1957). The activity was optimum at pH 7.4 and 37°C. The apparent K_m value was 14 μ M. The apparent K_m values for the dealkylation of several drugs by rat liver microsomal preparations (Rubin *et al.*, 1964) are around 10⁻⁴–10⁻³ M, i.e. several orders higher than the value with chlorfenvinphos. It is also significant that none of the potential competitive inhibitors (Table 2) added to the reaction mixtures greatly affected the rate of breakdown of chlorfenvinphos.

Oxidative *O*- and *N*-dealkylation reactions yield, not the respective alkanol, but the aldehyde; and the reaction is probably effected via hydroxylation of the α -carbon atom of the alkoxy group. If this mechanism is in operation in the de-ethylation of chlorfenvinphos, the ethyl group should be removed as acetaldehyde. The isolation of the [¹⁴C]acetaldehyde

Scheme 2. *Mechanism of dealkylation of chlorfenvinphos*

derivative from [¹⁴C]ethyl-labelled chlorfenvinphos thus also proves that the reaction is an oxidative dealkylation. In addition 2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate yields formaldehyde. The co-factor requirements, the site and the products of the reaction suggest that the apparently hydrolytic process is an oxidative one. The likely mechanism for the dealkylation of chlorfenvinphos is shown in Scheme 2. Hydroxylation of an α -carbon atom of an ethyl group would yield the unstable hemiacetal of acetaldehyde, which would break down immediately to the observed products. Dealkylation is probably a general reaction of this enzyme preparation, provided that the α -carbon atom of the alkyl group possesses a replaceable hydrogen atom. Diethyl and diisopropyl naphthyl phosphates were dealkylated at similar rates by the enzyme preparation.

The hepatic microsomal mixed-function oxidase system is clearly very important in the detoxification of chlorfenvinphos and possibly many other insecticidal phosphoric acid triesters in mammals, therefore the wealth of information on the effects of inhibition and stimulation (Mannering, 1968) of these enzymes must be brought into any considerations of the toxicology of the organophosphorus compounds.

Requests for reprints should be addressed to D. H. H.

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