Phosphoric Acid Triester–Glutathione Alkyltransferase

A MECHANISM FOR THE DETOXIFICATION OF DIMETHYL PHOSPHATE TRIESTERS

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1. 2-Chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate (tetrachlorvinphos) is demethylated by mammalian liver supernatant (100000g) protein in the presence of GSH. 2. GSH acts as an acceptor of the transferred methyl group to form *S*-methyl glutathione. 3. The enzyme that catalyses this reaction is present in the soluble fraction of liver from mouse, rat, rabbit and pig at similar activity. The enzyme was purified 45-fold from pig liver, dimethyl 1-naphthyl phosphate being used as assay substrate. 4. Methyl groups are readily removed from most of the substrates studied; ethyl groups are removed at one-fiftieth to one-hundredth of the rate for methyl groups. It is likely that the enzyme plays an important role in the detoxification of the phosphate triester pesticides containing $\text{CH}_3\text{--O--P}$ groups.

Insecticidal phosphoric acid triesters of the type shown in formula (I) (R = alkyl, X = electron-withdrawing substituent) may be detoxified via two routes: (i) removal of OX (esterase action) and (ii) removal of R or RO . We have reported results (Donninger *et al.*, 1967, 1972) proving that reaction (ii) predominated in the degradation of chlorfenvinphos [2-chloro-(2,4-dichlorophenyl)vinyl diethyl phosphate] by mammalian liver homogenates. It was shown that the reaction occurs in the microsomal fraction of rabbit liver homogenate and requires molecular oxygen and NADPH. Demethylation of tetrachlorvinphos [2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate, *cis* isomer] was shown to occur with the same enzyme preparation, but demethylation also occurred in the soluble fraction of the liver homogenate. The rate of demethylation by the soluble fraction was not affected by further centrifugation at 100000g for 2 h. Thus another type of demethylation, unconnected with the microsomal system, occurs in rabbit liver. We have investigated this enzyme in some detail as part of our study of the detoxification of phosphoric acid triesters. The present paper describes our investigations of the enzyme, its distribution, isolation, purification and substrate specificity. Some of the work has been published as a communication to the Biochemical Society (Hutson *et al.*, 1968).

Experimental

Materials

2-Chloro-1-(2,4,5-trichlorophenyl) [^{14}C]vinyl dimethyl phosphate (tetrachlorvinphos). We are indebted to Shell Development Company, Modesto, Calif., U.S.A., for synthesizing this material. It was purified before use by t.l.c. as described by Akintonwa & Hutson (1967).

^{14}C -labelling of one methyl group of tetrachlorvinphos. 2-Chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate (56g) was demethylated with sodium iodide in acetone as described by Whetstone *et al.* (1966) to give 35g of 2-chloro-1-(2,4,5-trichlorophenyl)vinyl methyl hydrogen phosphate (m.p. 137–138°C). This material (2.7g) was dissolved in acetone, and was evaporated to an oil and then dissolved in 40ml of sodium-dried ether. To this solution, 0.8g of dicyclohexylcarbodi-imide was added, and after 15 min the solution was evaporated to 30ml and the precipitated dicyclohexylurea was removed by filtration. The solution was cooled to 2°C for 16 h to yield *di*-[2-chloro-1-(2,4,5-trichlorophenyl)vinyl] dimethyl pyrophosphate, yield 1.3g (46%), m.p. 128–129°C (Found: C, 31.6; H, 1.8; Cl, 41.3; P, 9.2. $\text{C}_{18}\text{H}_{12}\text{O}_7\text{Cl}_2\text{P}_2$ requires: C, 31.5; H, 1.8; Cl, 41.4; P, 9.0%). The pyrophosphate (100mg) was dissolved by warming in 0.5ml of AnalaR acetone and the solution was introduced into a tube of [^{14}C]methanol (100 μCi ; 9.2mCi/mmol) (The Radiochemical Centre, Amersham, Bucks., U.K.), followed by 10 μl of carrier methanol. Sodium (2mg) was introduced into the tube with shaking and the solution was left for 30 min at room temperature. The mixture was then applied to two 20cm $\times$ 2mm prepared silica-gel plates

(E. Merck A.-G., Darmstadt, Germany), which were developed with hexane–acetone (7:2, v/v). The product (R_F 0.3) was located by scanning for radioactivity and was eluted with acetone. This solution was mixed with 10 ml of methanol and evaporated to dryness with methanol to ensure that no contamination with [^{14}C]methanol was occurring. Total radioactivity (61 μCi) was related to the total yield of product as determined by g.l.c. (7.0 mg), and the specific radioactivity was calculated to be 3180 $\mu\text{Ci}/\text{mmol}$. Conditions for g.l.c. were 0.91 m (3 ft) column of Oronite on Gaschrom-Q (80–100 mesh), pressure 172 kN $\cdot$ m $^{-2}$ (25 lb/in 2), at 190°C.

Dialkyl aryl phosphates: general method. Phenol or 1-naphthol (0.125 mol) was dissolved in a solution containing 0.13 mol of NaOH in 30 ml of water. Dialkyl phosphoryl chloride (0.1 mol) was added and the reaction mixture was shaken for 1 h. The mixture was then diluted with 500 ml of 2% (w/v) NaOH and the product was extracted with 500 ml of chloroform. The extract was washed with 2% NaOH until the washings were free of phenolic material, as indicated by the Gibbs reagent (see 'General methods'). Finally, the solution was washed with water, dried with anhydrous Na_2SO_4 and evaporated to a syrup. Yields were 60–70% and the products shown to be pure by t.l.c.

Dialkyl aryl phosphorothionates: general method. These compounds were prepared as described above by using dialkyl phosphorothionylchlorides and a reaction temperature of 60°C. Hexane was used for extraction of the products. Identities of all products were confirmed by n.m.r. spectroscopy.

Dialkyl [^{14}C]aryl phosphates and phosphorothionates. The radioactive compounds were prepared as described above, but on a 1.0 or 0.5 mmol scale. [^{14}C]Phenol and [^{14}C]1-naphthol were purchased from The Radiochemical Centre, and diluted with non-radioactive carrier material such that specific radioactivities were 50–100 $\mu\text{Ci}/\text{mmol}$. The ^{14}C -labelled products were isolated by preparative t.l.c.

***OS*-Dimethyl 1-naphthyl phosphorothiolate.** Dimethyl 1-naphthyl phosphorothionate was demethylated with trimethylamine in acetone to afford a 72% yield of crystalline tetramethylammonium methyl 1-naphthyl phosphorothionate, m.p. 109–113°C (Found: C, 55.3; H, 6.7; N, 3.9; P, 9.6; Calc. for $\text{C}_{15}\text{H}_{22}\text{O}_3\text{NPS}$: C, 55.0; H, 6.8; N, 4.3; P, 9.5%). This salt (0.176 mmol) was dissolved in methanol (0.25 ml) and was injected into a tube containing 100 μCi of [^{14}C]methyl iodide (30 mCi/mmol) and 22 μl of carrier methyl iodide. After 3 h the product was isolated by t.l.c. (CHCl_3 solvent) in 50% yield. The small-scale radioactive synthesis was preceded by a larger-scale non-radioactive synthesis (yields 90%) when the product was identified by its characteristic n.m.r. spectrum. *O*-Methyl-*S*-[^{14}C]methyl 1-naphthyl phosphorothiolate so prepared had a specific radioactivity of 280 $\mu\text{Ci}/\text{mmol}$.

***S*-Methylglutathione.** GSH (3.07 g, 10 mmol) and NaOH (1.2 g, 30 mmol) were dissolved together in 25 ml of water and 1.7 g (12 mmol) of methyl iodide was added. The mixture was made into one phase by the addition of 10 ml of methanol. The reaction mixture was analysed by paper chromatography on Whatman no. 1 paper with butan-1-ol–acetic acid–water (11:4:5, by vol.) as solvent. Conversion of GSH (R_F 0.40) into *S*-methylglutathione (R_F 0.45) was complete after a reaction time of 1 h. The solution was evaporated to remove methanol and methyl iodide, then was adjusted to pH 3.0 with 2M-HCl and evaporated to 30 ml. Ethanol (5 vol.) was added and the crystalline product was isolated after being left for 3 days at 2°C. The product was recrystallized by dissolving it in 10 ml of water and adding 50 ml of ethanol, to afford a 2.1 g (66%) yield, m.p. 202–204°C (decomp.). Kermack & Matheson (1957) reported m.p. 197–199°C (decomp.).

General methods

Paper chromatography. The descending technique was used, with Whatman no. 1 paper and butan-1-ol–acetic acid–water (11:4:5, by vol.). This solvent is the one of choice for separating *S*-methyl glutathione (R_F 0.45) from GSH (R_F 0.40) (Johnson, 1966). Ninhydrin and u.v. light were used to detect the metabolites.

Thin-layer chromatography. Merck prepared plates of silica gel F $_{254}$ were developed with hexane–acetone (7:2, v/v) or chloroform. Compounds were located by viewing under u.v. light, scanning for radioactivity or spraying with 2M-NaOH followed by the Gibbs reagent, 2,6-dichloro-*p*-benzoquinone-4-chloroimine (0.05%, w/v), in ethanol.

Assay of radioactivity. Samples were assayed by using a dioxan-based scintillator (Bray, 1960) in a Packard model 3003 Tri-Carb liquid-scintillation spectrometer. Radioactivity on chromatograms was located by scintillation counting of paper strips or silica gel from plates, or by scanning with a Desaga 12-2 gas-flow scanner.

Enzyme techniques

Enzyme assay. Typical enzyme assays were done as follows. An 0.4 mM solution of phosphate triester, ^{14}C -labelled in the vinyl or naphthyl groups, in chloroform or hexane (1 ml) was evaporated to dryness in a 25-ml Erlenmeyer flask. GSH or other co-factors dissolved in 2 ml of 0.1 M-potassium phosphate buffer, pH 7.4, was added and the reaction was started by the addition of 2 ml of enzyme solution (protein concn. 0.2–2 mg/ml). The mixture was incubated in a shaking water bath at 37°C; at intervals 1 ml samples were withdrawn and rapidly transferred to a mixture of 5 ml of benzene and 4 ml of

water, which was then vigorously shaken for 15s. After centrifugation, 1 ml of each layer was assayed for radioactivity. Dealkylated substrate (phosphate diester) remained in the aqueous phase and unchanged substrate partitioned almost completely (99%) into the benzene phase. In control reactions, the proportion of substrate remaining in the aqueous phase (0.1–1.0%) was a characteristic of the substrate and was constant over the concentration ranges chosen for these reactions.

Isolation of crude enzyme from liver. (i) Small-scale isolation. Liver (5g) from rat was homogenized in a Potter-Elvehjem type pestle and mortar (Aldridge *et al.*, 1960) with 30 ml of 0.1 M-potassium phosphate buffer and then was centrifuged at 100000g for 1 h. The supernatant was made 35% saturated with ammonium sulphate, then was centrifuged, and the resulting supernatant adjusted to 75% saturation with ammonium sulphate. The precipitate was dissolved in 0.1 M-potassium phosphate buffer and dialysed in this buffer. Protein concentrations were measured by a modification of the biuret reaction (Robinson & Hogden, 1940) or by the method of Lowry *et al.* (1951). The crude enzyme was stable to freezing and thawing and was stored at -20°C until used. All operations were done below 4°C .

(ii) Large-scale isolation and purification of enzyme from pig liver. Pig liver (800g) was homogenized in a domestic mincer and stirred with 2 litres of 0.1 M-potassium phosphate buffer, pH 7.4, for 1 h. During the first 5 min of this treatment, the mixture was intermittently treated with an Ultra-Turrax TP18 homogenizer. The homogenate (fraction P1) was filtered through muslin and was centrifuged for 1 h at 100000g to give the soluble supernatant (fraction P2). The protein was fractionated with ammonium sulphate and the fraction precipitating between 35 and 75% saturation was dissolved in 0.01 M-tris-HCl buffer, pH 7.4, and dialysed against this buffer for 16 h (fraction P5). The crude enzyme was contained in a volume of 700 ml at a protein concentration of 48 mg/ml. The activity was more stable in this preparation than that in the original homogenate. The next procedure, described below, is based on that used by Johnson (1966). The solution was diluted with 300 ml

of 0.1 M-tris-HCl buffer, pH 7.4, and cooled to -2°C . To this solution, 200 ml of aq. 90% (v/v) ethanol, previously cooled to -10°C , was added slowly with stirring. The resulting precipitate was removed by centrifugation at 5000g (at -5°C) and the supernatant treated with 12 ml of 2 M- ZnSO_4 . During these operations the pH of the system was adjusted to 7.4 by the dropwise addition of 1 M-tris, and was not allowed to fall below pH 6.5. After stirring for 20 min, the mixture was centrifuged and the precipitate, containing the active enzyme, was collected and immediately suspended in 0.01 M-tris-HCl buffer, and solubilized by the dropwise addition of 10% (w/v) EDTA adjusted to pH 7.4 with tris. The protein was recovered by saturating the solution to 75% with ammonium sulphate and dialysing the resultant precipitate against 0.01 M-potassium phosphate buffer. At this stage, the enzyme was contained in 270 ml of solution at 34 mg of protein/ml (fraction P7).

The solution was diluted with 500 ml of water and stirred with 150 ml of calcium phosphate gel (3.0%, w/v, suspension in water; BDH Chemicals Ltd., Poole, Dorset, U.K.). After centrifugation, the gel and adsorbed protein were discarded and the supernatant (fraction P8) (810 ml, 5.2 mg of protein/ml) was fractionated with ammonium sulphate. The active fraction precipitating between 45 and 65% saturation was collected and redissolved in 0.1 M-potassium phosphate buffer to yield 99 ml of solution containing 25.6 mg of protein/ml (fraction P9). The solution was stored in 5 ml portions at -20°C until required for use or for further purification.

(iii) Gel filtration of the enzyme on Sephadex G-150. After small-scale experiments the following procedure was devised for further purification of the enzyme. Sephadex G-150 [Pharmacia (GB) Ltd., London W.13, U.K.] was preswollen in 0.05 M-potassium phosphate buffer, pH 7.4, degassed, and poured into a Pharmacia K25/100 column to give a gel column $2.5\text{ cm} \times 95\text{ cm}$. The column was operated at a hydrostatic pressure of $981\text{ N}\cdot\text{m}^{-2}$. The void volume (V_0) of the column was determined by passing 3 ml of 0.1% Blue Dextran 2000 down the column. Two columns made in the same way had the same values (void volume, 160 ml; total volume, 470 ml).

(II)

(III)

Chromatography was done by applying 5 ml of fraction P9 (115 mg of protein) to the top of the column and eluting the column with 500 ml of 0.05 M-potassium phosphate buffer, 8–12 ml fractions being collected. Protein concentrations in the fractions were determined by the Lowry *et al.* (1951) method, and enzyme assays were done by the standard procedure scaled down to 2 ml (1 ml of GSH solution, 0.5 ml of buffer and 0.5 ml of column fraction) and incubated for 10 min. The results of such a fractionation were reproducible for two different columns and five runs on each one gave an elution volume (V_e) for the enzyme peak of 340–350 ml. The active enzyme fractions (between 320 and 360 ml, containing 49% of the activity applied to the column at a specific activity of 21 units/mg) were combined and concentrated five-fold by ultra-filtration in an Amicon model 50 cell fitted with a PM-30 membrane, operating at $172 \text{ kN} \cdot \text{m}^{-2}$ of N_2 (Amicon NV, Oosterhout, Holland).

Results

Initially our investigations were confined to the demethylation of the phosphate triester 2-chloro-1-(2,4,5-trichlorophenyl) [^{14}C]vinyl dimethyl phosphate (formula II, referred to hereafter as tetrachlorvinphos).

Location and nature of the enzyme activity

When [^{14}C]tetrachlorvinphos was incubated with the soluble fraction of rabbit liver homogenate it was degraded to a water-soluble metabolite that behaved as 2-chloro-1-(2,4,5-trichlorophenyl)vinyl methyl hydrogen phosphate (formula III) on paper chromatography. The enzyme activity was destroyed by boiling, but not by freezing and thawing. Activity was lost on dialysis but regained on addition of GSH. The rates of demethylation of tetrachlorvinphos by rabbit liver microsomal fraction (Donninger *et al.*, 1972) and soluble fraction were compared and the absolute values were: microsomal fraction, 1.1; soluble fraction, 3.0 (nmol converted/min per mg of protein). Activity in the soluble fraction was not diminished by centrifugation for a further 2 h. Therefore a soluble enzyme with certain cofactor requirements was responsible for the demethylation of the phosphoric acid triester. Two possibly related enzyme systems were reported in the literature. Johnson (1966) described a soluble enzyme from rat liver that catalysed the breakdown of methyl iodide in the presence of GSH and Fukami & Shishido (1966) described an enzyme from the same source, also GSH-dependent, which catalysed the demethylation of methylparathion. A study of the cofactor requirements was therefore done. The results are shown in Table 1, and indicate that GSH is probably a specific

Table 1. *Cofactor requirements of the enzyme catalysing the degradation of tetrachlorvinphos*

Rabbit liver soluble fraction was dialysed against 0.01 M-potassium phosphate buffer, pH 7.4, and assayed at a protein concentration of 1 mg/ml with 2-chloro-1-(2,4,5-trichlorophenyl)[^{14}C]vinyl dimethyl phosphate (tetrachlorvinphos) (0.05 mM) as a substrate.

Factors	Conversion of substrate (nmol/min per mg of protein)
Undialysed protein	3.00
None	0.04
NADPH-generating system	0.04
GSH (5 mM)	2.25
GSH (0.5 mM)	0.95
GSH (0.05 mM)	0.20
Cysteine (5 mM)	0.04
Mercaptoethanol (5 mM)	0.10

cofactor for the enzyme. Cysteine, for example, has no effect as a cofactor. Johnson (1966) described the methyl iodide-degrading enzyme as a glutathione *S*-alkyltransferase, and demonstrated by a chromatographic method that GSH was converted into *S*-methyl glutathione during the enzymic reaction. This aspect had not been examined in the phosphoric acid triester field.

Products of the reaction

2-Chloro-1-(2,4,5-trichlorophenyl)vinyl di-[^{14}C]methyl phosphate (^{14}C -methyl-labelled tetrachlorvinphos) (0.2 mM) was incubated with crude enzyme from rabbit liver (1 mg of protein/ml) and GSH (5 mM). After 30 min 35% of the substrate was converted into water-soluble product. The unchanged substrate was removed by hexane extraction and the water-soluble products were analysed by paper chromatography. Radioactivity was found to co-incide with *S*-methylglutathione (R_f 0.45) and 2-chloro-1-(2,4,5-trichlorophenyl)vinyl methyl hydrogen phosphate (R_f 0.9). The separation of radioactivity and location of authentic marker compounds are shown in Fig. 1. To confirm the chromatographic assignments, part of the aqueous fraction after hexane extraction was analysed by isotope-dilution analysis with both *S*-methylglutathione and the hydrogen phosphate derivative (compound III). The carrier compounds were isolated by crystallization from ethanol–water (5:1, v/v) and dichloromethane respectively. Of the water-soluble radioactivity, 50.3% was due to the hydrogen phosphate metabolite and 40.2% to *S*-methylglutathione.

Species distribution of the enzyme

Livers were obtained from animals killed by a blow on the head, except for the pig, when livers were obtained from an abattoir. Soluble fractions of the livers were prepared, dialysed, adjusted to a concentration of 1 mg of protein/ml, and GSH was added to give a final concentration of 5 mM. Enzyme activities were assayed by the standard method, by using three

measurements between 0 and 15 min. The results are shown in Table 2. The four species studied possess similar enzyme activities.

Substrate specificity of the enzyme

The solubility of tetrachlorvinphos is 0.03 mM in water at 25°C and, in the work described above, the

Fig. 1. Paper chromatography of the products of demethylation of 2-chloro-1-(2,4,5-trichlorophenyl)vinyl di-[^{14}C]-methyl phosphate

[^{14}C]Methyl-labelled tetrachlorvinphos (0.2 mM) was incubated with crude enzyme from rabbit liver (1 mg of protein/ml) and GSH (5 mM). After removal of unchanged substrate by hexane extraction, the aqueous phase was analysed by paper chromatography in the butanol-acetic acid solvent. The positions of marker compounds are shown under the base line, and distribution of radioactivity above the base line. GSMe, *S*-methylglutathione; Des-Me substrate, 2-chloro-1-(2,4,5-trichlorophenyl)vinyl methyl hydrogen phosphate (compound III); GSSG, oxidized glutathione. The radioactive products were found, by isotope-dilution analysis, to consist of *S*-methylglutathione (40.2%) and compound (III) (50.3%).

Table 2. Species variation in the demethylation of tetrachlorvinphos

Results are expressed as nmol of substrate converted/min per mg of protein and are initial-rate measurements determined graphically from three duplicate points. Tetrachlorvinphos was used as substrate except for the pig, when assays were done with dimethyl 1-naphthyl phosphate as substrate. This compound is demethylated at about 0.25 times the rate of tetrachlorvinphos in rabbit liver supernatant at this substrate concentration. The GSH concentration was 5 mM.

Species	Values from individual animals						Mean	S.E.M.
Mouse	1.20	1.86	1.04	1.62	1.62	1.72	1.51	± 0.13
Rat	2.60	1.90	1.90	1.70			2.03	± 0.20
Rabbit	2.40	1.90	2.00				2.10	± 0.153
Pig	1.05	1.32					1.19	—

soluble protein in the incubation mixtures (1 mg/ml) aided its dissolution. However, the insolubility of the substrate placed two limitations on further experiments: (i) it was not possible to increase the substrate concentration for kinetic studies; (ii) purification of the enzyme would lead to lower protein concentrations in the assay solutions, and thus to incomplete dissolution of the substrate.

Several potential ^{14}C -labelled substrates were therefore synthesized with a view to (i) finding a conveniently soluble substrate and (ii) examining the substrate specificity of the reaction. The following series of phenyl and 1-naphthyl dialkyl phosphates and phosphorothionates were prepared, containing [^{14}C]aryl moieties: dimethyl phenyl phosphate (50 $\mu\text{Ci}/\text{mmol}$), dimethyl phenyl phosphorothionate (65.8 $\mu\text{Ci}/\text{mmol}$), dimethyl 1-naphthyl phosphate (71.4 $\mu\text{Ci}/\text{mmol}$), diethyl 1-naphthyl phosphate (71.4 $\mu\text{Ci}/\text{mmol}$) and di-isopropyl 1-naphthyl phosphate (71.4 $\mu\text{Ci}/\text{mmol}$). The substrates were incubated with crude enzyme, and the rates of dealkylation are shown in Table 3. Methyl groups are rapidly removed from most of the dimethyl phosphoric acid triesters, but ethyl and isopropyl groups are removed only slowly. By using high protein concentrations and long incubation periods we were able to demonstrate, by paper chromatography, the de-ethylation of 2-chloro-1-(2,4-dichlorophenyl)vinyl [^{14}C]diethyl phosphate (chlorfenvinphos) and the formation of [^{14}C]ethylglutathione.

Removal of S-methyl groups relative to O-methyl groups

A racemic mixture of *O*-methyl-*S*-[^{14}C]methyl 1-naphthyl phosphorothiolate was incubated with crude enzyme under standard conditions, and after

benzene-water partition and assay of radioactivity, the water-soluble products were examined by the chromatographic method described above. No radioactivity was detected in the *S*-methylglutathione area of the chromatogram and the only detectable radioactivity was at R_F 0.9, the position of authentic *S*-methyl 1-naphthyl hydrogen phosphorothiolate. This indicated that only *O*-methyl groups were removed from this substrate.

Dimethyl 1-[^{14}C]naphthyl phosphate as substrate

The solubility and rate of demethylation of dimethyl 1-naphthyl phosphate made this triester a suitable substrate and all further work was done with it as the standard substrate for enzyme assay. The rates of demethylation of the substrate were proportional to protein concentrations up to 1.5 mg/ml. The rates were linear at a substrate concentration of 0.1 mM if incubation times were such that no more than 30% of the substrate was demethylated. At a substrate concentration of 0.1 mM, the maximum rates of reaction were obtained at a GSH concentration of 3 mM and above. A concentration of 5 mM was used thereafter. The pH optimum of the rabbit liver enzyme was determined at this stage, and the pH-activity curve was bell-shaped, with the optimum between pH 7 and 8.5.

Purification and properties of the enzyme from pig liver

The enzyme was isolated from pig liver and partially purified by the methods described in the Experimental section. The K_D value, the distribution constant for the enzyme between Sephadex G-150 and the buffer, was 0.41. The most active fraction from the final gel-filtration stage represented a 46-fold puri-

Table 3. *Rates of dealkylation of some phosphate triesters*

Rabbit liver 100000g supernatant (precipitated between 35 and 75% -satd. ammonium sulphate) was incubated at 37°C at a concentration of 1 mg of protein/ml in 0.1 M-potassium phosphate buffer, pH 7.4, with substrate (0.1 mM) and glutathione (5 mM). Conversion of substrate into product was measured by the radioassay procedure described in the Experimental section.

Substrate	Rate of dealkylation (nmol/min per mg of protein)
Dimethyl phenyl phosphate	0.08
Dimethyl phenyl phosphorothionate	0.60
Dimethyl 1-naphthyl phosphate	0.66
Dimethyl 1-naphthyl phosphorothionate	1.90
Dimethyl 1-naphthyl phosphorothiolate	0.39
2-Chloro-1-(2,5-dichlorophenyl)vinyl dimethyl phosphate	2.55
2-Chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate	2.60
2-Chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate	0.03
Diethyl 1-naphthyl phosphate	0.01
Di-isopropyl 1-naphthyl phosphate	0.02

Table 4. *Purification of the enzyme from pig liver*

Enzyme assays were done as described in the Experimental section with dimethyl 1- $[^{14}\text{C}]$ naphthyl phosphate as substrate. One unit of enzyme activity is defined as the amount that catalyses the conversion of 1 nmol of substrate/min under the standard conditions.

Fraction	Volume (ml)	Concn. of protein (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Recovery (%)
P1 (Homogenate)	1760	152	267 520	0.66	176 563	100
P2 (100000g supernatant)	1140	—	—	—	—	—
P3 (35% ammonium sulphate precipitate)	250	47	12 100	0.60	7260	4
P4 (0→35% ammonium sulphate supernatant)	1100	—	—	—	—	—
P5 (35→75% ammonium sulphate precipitate)	700	48	33 600	2.2	73 920	42
P6 (75% ammonium sulphate supernatant)	1200	<0.2	<240	0	0	—
P7 (After Zn-ethanol precipitation, dialysed)	270	34	9180	5.6	51 408	29
P8 (Calcium phosphate-gel-treated)	810	5.2	4212	7.4	31 169	18
P9 (45→65% ammonium sulphate precipitate)	99	25.6	2534	8.0	20 272	12
P10 (Active fraction after Sephadex G-150 filtration of part of P9. The recovery figure assumes that all of P9 was so treated)	—	—	—	21.0	—	6

fication from the original homogenate. The pooled fractions generally were 32-fold purified. The progress of the purification is summarized in Table 4.

Demethylation by pig liver enzyme followed approximately the same pattern as with rabbit liver enzyme. The effects of a 13-fold purification (fractions P2 to P9) on the specificity is shown in Table 5. These results should not be regarded as true rates in every case, because in some cases the uptake of the less-soluble substrates actually limited the rate of demethylation. The ratios of enzyme activities of fractions P9 and P2 also indicate that the fraction P9 sample had lost some activity on storage at -20°C (cf. Table 4).

Kinetics. With a GSH concentration of 5 mM and enzyme purified by gel filtration (fraction P10), the initial rates of demethylation of dimethyl 1-naphthyl phosphate at various substrate concentrations were measured. One example of the results is shown as a Lineweaver & Burk (1934) plot in Fig. 2. The apparent K_m for the system was calculated from five sets of results and was 0.16 mM (0.13–0.20 mM, 95% confidence limits).

Methyl iodide-glutathione methyltransferase

This enzyme was reported by Johnson (1966) who studied it in rat liver. Incubation of our pig liver enzyme fractions with GSH (5 mM) and $[^{14}\text{C}]$ methyl iodide and analysis of the non-volatile water-soluble

radioactive material confirmed that this enzyme was present in pig liver supernatant. Appropriate control reactions indicated a slow reproducible non-enzymic reaction of methyl iodide with GSH. There was no detectable non-enzymic reaction of methyl iodide with protein. The relative rates of demethylation of methyl iodide and dimethyl 1-naphthyl phosphate were measured. The absolute rates (nmol/min per mg of protein) for crude pig liver enzyme were 11.8 and 0.73 respectively. This was repeated with gel-filtered enzyme to give values of 276 and 16.4 respectively. The ratios of the rates with crude enzyme (16.2) and with 20-fold-purified gel-filtered enzyme (16.8) shows that the two enzyme activities are purified to the same extent by the methods used. This would suggest that one enzyme may be responsible for both reactions.

Discussion

Mammalian liver contains a soluble enzyme capable of degrading the $\text{CH}_3\text{-O-P}$ bond in dimethyl phosphoric acid triesters or dimethyl phosphorothionic acid triesters. GSH is a specific cofactor for this enzyme and we have shown that one of its roles is that of acceptor of the methyl group. The reaction is thus a transferase type in which the enzyme catalyses the transfer of a methyl group from a donor (phosphate triester) to the acceptor (GSH); the enzyme can be called *phosphoric acid triester-glutathione*

Table 5. Rates of demethylation of some substrates with pig liver enzymes

The enzyme preparations are described in Table 4. Assays were done as described in the Experimental section; substrate concentration 0.1 mM. Dissolution of substrates indicated by * was very poor and rate-limiting. This accounts for the very low rate of demethylation of tetrachlorvinphos when compared with rabbit liver crude enzyme.

Substrate	Demethylation (nmol/min per mg of protein)		Ratio of activity P9/P2
	Crude enzyme (P2)	Partially purified enzyme (P9)	
Dimethyl 1-naphthyl phosphate	0.73	6.71	9.2
Dimethyl 1-naphthyl phosphorothionate*	0.69	4.20	6.1
Dimethyl phenyl phosphate	0.36	2.94	8.2
Dimethyl phenyl phosphorothionate	1.20	6.01	5.0
Diethyl 1-naphthyl phosphate	0.01	0.06	6.0
2-Chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate (tetrachlorvinphos)*	—	0.98	—
2-Chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate	—	0.03	—

Fig. 2. Lineweaver-Burk plot for the demethylation of dimethyl 1-naphthyl phosphate with purified pig liver enzyme (fraction P10)

The glutathione concentration was 5 mM, and the concentration of the substrate was varied. One set of results is illustrated. The apparent K_m calculated from five sets of results was 0.16 mM. R is the rate of demethylation (nmol/min), and $[S]$ is the substrate concn. (mM).

S-alkyltransferase. Ethyl and isopropyl groups are transferred but at very low rates (approx. 1/50 of that for methyl groups). *S*-Methyl groups are not readily transferred, and in the presence of an *O*-methyl group in the same molecule the latter is removed preferenti-

ally. The pattern of rates of transfer approximately parallels the chemical reactivity of the various bonds in chemical dealkylations of phosphoric acid triesters by nucleophiles, e.g. trimethylamine and thiophenol (Miller, 1962). The reaction may be regarded as an enzymically catalysed alkylation of GSH.

There are several GSH-dependent enzymes that are important in the detoxification of drugs and insecticides (Boyland & Chasseaud, 1969a): GSH *S*-alkyltransferase (Johnson, 1966), GSH *S*-aryltransferase (Grover & Sims, 1964), GSH *S*-epoxidotransferase (Boyland & Williams, 1965), GSH *S*-alkenyltransferase (Boyland & Chasseaud, 1968) and GSH alkaryltransferase (Boyland & Chasseaud, 1969b). We have not undertaken comparative studies in the present work but during the purification of the enzyme, the ratios of the rates of transfer from methyl iodide and from dimethyl naphthyl phosphate were the same at two stages. The two reactions may possibly be catalysed by the same enzyme.

Thus we have found two enzyme systems in mammalian liver that demethylate 2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate and other dimethyl phosphate triesters. We have shown (Donninger *et al.*, 1967, 1972) that dealkylation by the hepatic microsomal mono-oxygenase system occurs readily with methyl, ethyl and isopropyl groups, but wide variations (about 100-fold) in the activities of the microsomal enzyme occur from species to species. The soluble methyl-transferring enzyme is present at similar activities in the liver of rat, mouse, rabbit and pig, but is relatively specific for the removal of methyl groups. We have not investigated the relative roles of the two mechanisms of detoxification *in vivo*,

but it has now been demonstrated by Hollingworth (1969) that, when mice are treated with dimethyl 3-methyl-4-nitrophenyl phosphate and phosphorothioate, there is a considerable depletion of liver GSH, which returns to normal values after 12h. This is an indication that the system operates *in vivo*. This author has also identified *S*-methylglutathione as the product of enzyme action *in vitro*. The enzyme is probably also involved in the detoxification of the insecticide dimethyl 2,2-dichlorovinyl phosphate (dichlorvos) (Dicowsky & Morello, 1971; Hutson *et al.*, 1972). The GSH-dependent demethylation of dimethyl 1,3-dicarbomethoxy-1-propen-2-yl phosphate and mevinphos (dimethyl-1-carbomethoxy-prop-1-en-2-yl phosphate) in mouse liver has been reported (Morello *et al.*, 1968). These authors also suggest that the enzyme plays an important role in the detoxification of organophosphates but that GSH concentration in the liver may be a limiting factor. In addition, it was shown that the *cis* isomer of mevinphos only is degraded by this route, the *trans* isomer being cleaved at the P-O-vinyl bond. The contribution of the two systems to detoxification will depend on the structure of the particular pesticide in question.

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