Dealkylation of Organophosphorus Esters by Mouse Liver Enzymes in Vitro and in Vivo

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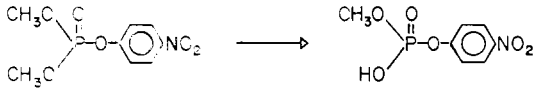
The mechanism and relative importance of *O*-dealkylation in detoxication of insecticidal organophosphate esters were investigated in white mice. The products of *O*-dealkylation of methyl paraoxon in liver supernatants were identified as demethyl methyl paraoxon and *S*-methylglutathione. Dimethyl esters were degraded by this enzyme much more readily than diethyl or diisopropyl esters. Liver microsomes with NADPH produced dimethyl phosphate. Little if any oxidative *O*-dealkylation occurred. Methyl paraoxon administered orally to mice gave dimethyl phosphate as the main urinary metabolite with lesser amounts of the demethyl derivative. No *S*-methylglutathione appeared in the urine because of further metabolism to volatile respirable compounds. The depletion of glutathione in the livers of Sumithion-treated mice and synergism of toxicity by methyl iodide suggest that, as in vitro, the major route of *O*-dealkylation in vivo is by alkylation of glutathione.

Although the dialkyl phosphoric acid triesters as insecticides have stimulated considerable commercial and scientific interest for many years, detailed information is not available on many of the mechanisms by which they are detoxified. Most metabolic investigations have been concerned with cleavage at the anhydride rather than alkyl groups (O'Brien, 1967; Terriere, 1968) and the term "hydrolysis" is still commonly used to cover all types of cleavage, although there is little mechanistic evidence for this view.

O-Dealkylation, yielding nontoxic dealkyl compounds as illustrated below, has been repeatedly noted as a detoxication mechanism of insecticidal organophosphate esters in mammals, insects, plants, and microorganisms.



Methyl paraoxon Demethyl methyl paraoxon

The relative contribution of *O*-dealkylation increases markedly with increasing dosage level of several organophosphates in rats (Plapp and Casida, 1958) and in mice though not in houseflies (Hollingworth *et al.*, 1967b), and at higher dosage levels may become the major means of detoxication in mammals. *O*-Dealkylation may also be a detoxication mechanism of importance in insects. In this respect, Kojima *et al.* (1963) attribute appearance of resistance against parathion in the rice stem borer to increased ability to dealkylate the insecticide and its oxidation product, paraoxon.

Fukami and Shishido (1963, 1966) examined the general properties of the *O*-dealkylating system in several mammalian and insect tissues with methyl parathion and related compounds. They concluded that the major site of detoxication in mammals was the liver and one of the predominant mechanisms in the liver was *O*-dealkylation. Glutathione was a required co-factor for *O*-dealkylation by both insect and mammalian tissue supernatants.

The aim of this investigation was to study further the mechanism of *O*-dealkylation of organophosphate esters and to examine the relative effectiveness of competing pathways of metabolism in vitro and in vivo.

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MATERIALS AND METHODS

$^{14}CH_3$-Labeled Methyl Paraoxon (Dimethyl *p*-Nitrophenyl Phosphate). *p*-Nitrophenyl phosphoric dichloride was prepared by the method of Tsuchiya *et al.* (1965). The product was distilled (b.p. at 0.5 mm. 137–42° C.) and, immediately before use in the following reaction, was recrystallized from *n*-hexane–chloroform (m.p. 43–45° C.; lit. 39–41° C.).

Methanol-^{14}C (2 mCi; specific activity 9.6 mCi per mmole; Nuclear Chicago Corp.) was transferred with 7 ml. of dry benzene to a borosilicate glass tube immersed in a dry ice freezing bath. Dry pyridine (158 mg.), a further 57 mg. of unlabeled methanol, and 256 mg. of dichloride in 5 ml. of benzene were added and the tube was sealed. After standing at room temperature for 1 hour with occasional shaking, the tube was cooled, opened, and the precipitate removed by filtration. The filtrate was quickly washed twice with 5% Na_2CO_3, twice with water, and dried over anhydrous sodium sulfate, and the solvent removed using a rotary evaporator. The residue was taken up in methanol, and transferred to a small tube, and the solvent removed by a gentle air stream followed by evacuation at 0.05 mm. of Hg for 1 hour.

The product (152 mg., 62% yield) was a clear, light yellow liquid with at least 98% radiochemical purity as judged by paper chromatography. No radioactive water-soluble impurities were present.

Other Organophosphate Esters. Methyl paraoxon, dimethyl 3-methyl-4-nitrophenyl phosphate (sumioxon), diethyl *p*-nitrophenyl phosphate (paraoxon), diisopropyl *p*-nitrophenyl phosphate (isopropyl paraoxon), and dimethyl 3-methyl-4-nitrophenyl phosphorothionate (Sumithion) were prepared as described by Hollingworth *et al.* (1967a). Ethyl dichlorvos (2,2-dichlorovinyl diethyl phosphate) was synthesized by the Perkow reaction (Barthel *et al.*, 1955). Dichlorvos (2,2-dichlorovinyl dimethyl phosphate) and chlorfenvinphos [2-chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate] were analytical grade samples obtained from the Shell Development Co., Modesto, Calif. With the exception of Sumithion and chlorfenvinphos, these compounds were finally purified by vacuum distillation and all had physical characteristics in good agreement with literature values.

Tris(1-aziridinyl)phosphine oxide (TEPA) was prepared by the reactions of Wystrach *et al.* (1955) and Bestian (1950). The hygroscopic product was recrystallized from *n*-hexane–benzene (m.p. 41–42° C.; lit. 41° C.).

Amino Acids and Derivatives. *S*-Methylglutathione (GSMe) was synthesized as described by Martin and Edsall (1958). A white crystalline product was obtained from

aqueous ethanol [m.p. 189–91.5° C. (decomp.); lit. 189° C.].

S-Methyl-L-cysteine (CySMe) was obtained from the Sigma Chemical Co., St. Louis, Mo.

S-(*N*-ethylsuccinimido)glutathione was prepared by the method of Lee and Samuels (1961). Repeated crystallization from aqueous ethanol gave a product with a melting point of 200.5–02° C. (decomp.), which was free of glutathione. Lee and Samuels (1961) give a melting point of 204–06° C. (decomp.).

Dinitrophenyl amino acids (DNP-amino acids) were obtained by the reaction of 2,4-dinitrofluorobenzene with the appropriate amino acid (Levy and Chung, 1955). DNP-GSMe was recrystallized several times from aqueous methanol (m.p. 147.5–49° C.). Acid hydrolysis of GSMe was carried out by dissolving 5 mg. of the standard compound or a metabolite fraction containing GSMe in 0.25 ml. of 6*N* HCl and heating to 110° C. for 8 hours under nitrogen in a sealed tube. The contents of the tube were then evaporated several times to dryness on a rotary evaporator, with the addition of a little distilled water each time, before the concentrated material was used for chromatography. This method caused little or no loss of CySMe, as shown by test runs with GSMe or CySMe itself.

Homogenates and Subcellular Fractions. Male, Swiss white mice, 3 to 6 months old, were killed with chloroform and their livers immediately removed and washed with ice-cold 0.9% NaCl. After homogenization in a cooled Duall tissue grinder with 10 times their weight of cold 0.1*M*, pH 7.4 phosphate buffer, the subcellular fractions were separated by centrifugation at 3° C. For preparation of microsomal and soluble fractions the homogenate was centrifuged at 10,000 × G for 10 minutes and the resulting sediment discarded. The supernatant from this stage was further centrifuged for 30 minutes at 177,000 × G_{max}. The microsomal pellet was washed once by suspension in buffer and recentrifugation and the final preparation made by resuspending the microsomes in the original volume of buffer. The solution obtained after removal of the microsomes is termed the supernatant fraction.

When mitrochondrial preparations were required, the homogenization and resuspension stages were carried out in 0.25*M* sucrose containing 10 m*M* potassium phosphate, pH 7.4. After an initial separation at 800 × G for 5 minutes, the mitochondrial pellet was obtained by centrifugation at 10,000 × G for 10 minutes and was washed and recentrifuged once. The supernatant above the mitochondria was then processed as described above to obtain microsomes and supernatant.

Incubation of Tissue Fractions. Incubations were carried out at 37° C., either in 16-ml. flasks on a Warburg respirometer or in 25-ml. open beakers in a Dubnoff metabolic shaker. No difference was observed between the two methods.

Except where noted, the incubation mixture contained 2.5 ml. of appropriate tissue fraction (equivalent to the tissue derived from 250 mg. of liver), 0.3 ml. of glutathione or other appropriate cofactor in buffer, 0.1 ml. of substrate in acetone, and 0.1 ml. of potential inhibitor in buffer when necessary. The final volume was 3.0 ml. Microsomal incubations were carried out as described, but in some cases nicotinamide (10 m*M*), semicarbazide (2 mg. per ml. of incubate), or magnesium chloride (10 m*M*) was also included as indicated. Since acetone inhibited microsomal degradation, the substrate was added in aqueous solution. When larger amounts of metabolites from the supernatant or microsomal incubations were needed, all volumes were increased fivefold.

After incubation, the solutions were deproteinized by making up to a 3% solution with trichloroacetic acid or, when the incubates were to be run on the gas chromatograph, by shaking for 1 minute in boiling water and immediately recooling. In either case the precipitate was removed by centrifugation, washed once, and recentrifuged, and the supernatants were combined for analysis.

Estimation of Total Degradation. A reasonably accurate estimate of the degree of degradation of the organophosphate substrates was possible by partitioning the deproteinized solutions three times against an equal volume of ether or chloroform. The intact organophosphate in the combined organic phases was quantitatively separated from the metabolites in the water phase. With radioactive substrates, the radioactivity in both aqueous and organic phases was counted with a Packard Tri-Carb liquid scintillation counter. For nonradioactive substrates, the residual organophosphate in in the organic layer was estimated by gas chromatography. After heat-deproteinization of the incubates, the organic-soluble material was suitably diluted with benzene and estimated with a Varian Aerograph Model 2000 gas chromatograph using a glass column (5 feet × 1/8 inch) packed with 3% Apiezon N on Chromosorb G, 60- to 80-mesh, acid-washed, and DMCS-treated (Nakatsugawa *et al.*, 1968). Column temperatures and nitrogen flow rates ranged from 135° C. and 57 ml. per minute for dichlorvos through 170° C. and 93 ml. per minute for TEPA to 210° C. and 93 ml. per minute for isopropyl paraoxon and chlorfenvinphos. Retention times ranged from 1.4 minutes for dichlorvos to 4.8 minutes for chlorfenvinphos. A flame photometric detector (Melpar, Inc., Falls Church, Va., Model 65-34A) with a 526-mμ filter was used. The high specificity of the detector for phosphorus made it possible to run the samples without preliminary cleanup. Before starting a run with the chromatograph, the system was conditioned by injecting several large samples of organophosphate until a constant response was observed. Standard solutions were run frequently to keep a check on reproducibility of the detector.

Separation and Estimation of Metabolites. A variety of chromatographic methods were used to identify and estimate the individual metabolites. The initial separation was generally by ion-exchange chromatography, sometimes supplemented by thin-layer chromatography.

The ion exchange separation was carried out directly on the aqueous phase from deproteinized incubates or on urine samples. The method was as described by Hollingworth *et al.* (1967b), except that only the first three elution gradients were used. The radioactive peaks were located and quantitated by scintillation counting and identified by cochromatography with authentic samples of potential metabolites. Identity was confirmed by color tests and by rechromatography of fractions from the eluate.

Thin-layer plates were prepared with cellulose MN300, 250 microns thick, and were air-dried after predevelopment with 0.1*N* HCl. The chromatograms were developed with 2-propanol–water–concentrated NH_4OH (75:24:1 by volume, system A).

The incubates themselves and the early fractions from the ion exchange column contained considerable amounts of salts which interfered with subsequent chromatographic separations. Thus, a simple desalting technique was used which greatly improved subsequent chromatography.

Dowex 1-X10 (200- to 400-mesh) anion exchange resin was shaken with carbonate-free 1*N* NaOH and then with water until the washings were neutral. A 2 × 4 cm. column of the resin was prepared and the solution to be desalted was added, followed by a first elution with 20 ml. of distilled water at a

flow rate of 0.3 ml. per minute. The water was followed by 15 ml. of 1*N* acetic acid and the acid front was observed by the color of the resin. The acid eluate containing the neutral and acidic amino acids was collected and evaporated several times to dryness, with the addition of small amounts of water each time before further chromatography. The completeness of elution by the acetic acid was occasionally checked by a final elution with 1*N* HCl, which showed that more than 99% of the radioactivity was in the acetic acid fraction.

Paper chromatography was carried out by the ascending technique on strips of Whatman No. 1 paper. Four solvent systems were used for amino acid separations,

System B. Phenol-water (4 to 1, w./v., freshly made)
System C. 1-Butanol–pyridine–concentrated NH_4OH–water (8:6:3:3, by volume)
System D. Chloroform-methanol–concentrated NH_4OH–water (4:4:1:1, by volume; upper phase taken)
System E. 1-Propanol–glacial acetic acid–water (14:3:3, by volume, freshly made)

Known and unknown DNP-amino acids were separated by two-dimensional chromatography on layers of silica gel G, 250 microns thick. The plates were dried at room temperature and developed first with 1-propanol–concentrated NH_4OH (4 to 1, by volume, system F) to a height of 12 cm. The plates were then dried in an air stream for 20 minutes, followed by 15 minutes at 60° C. After cooling to room temperature they were run in the second direction for 12 cm. with chloroform–ethyl acetate–formic acid (9:9:2 by volume, system G). The yellow spots were conveniently recorded on Polaroid Type 51 high contrast film.

Electrophoresis was carried out on Whatman 3MM strips (2 × 20 cm.) with an effective length of 15 cm. A constant gradient of 300 volts was applied. The runs were made in a cold room at 3° to 5° C. for 4 hours under a saturated atmosphere in a closed container. Electroosmosis was negligible as judged by the immobility of glucose.

Two buffers were used in electrophoresis.

System H. Glacial acetic acid–pyridine (10 to 1, by volume), 2 ml. added to 98 ml. of water; pH 3.6
System I. Glacial acetic acid–pyridine (49 to 1, by volume), 2 ml. added to 98 ml. of water; pH 3.0

Location and Identification of Metabolites. The radioactive areas on both chromatographic and electrophoretic strips were located by strip scanning (Nuclear Chicago Actigraph III) and autoradiography. Information on the nature and position of metabolites was obtained by specific color tests carried out on the column eluate fractions or the chromatographic strips.

Metabolites containing *p*-nitrophenol were detected by the yellow color produced on alkaline hydrolysis. Heating was sometimes necessary. Compounds containing phosphorus were revealed by the Hanes-Isherwood reagent. Amino acids were detected and quantitated in solution with ninhydrin. Amino acids containing sulfur were located with platinic iodide reagent or nitroprusside reagent where a free sulfhydryl group was present (Toennies and Kolb, 1951). A ninhydrin spray could be superimposed on the strips after the platinic iodide reaction had faded but there was some loss of sensitivity.

Metabolism of Methyl Paraoxon in Vivo. ^{14}C-Methyl paraoxon in olive oil was administered orally to male white mice (25 to 30 grams) at either 1 or 4 mg. per kg. Five mice were used at each dosage level and each was given 0.25 ml. of solution after a 6-hour starvation period. They were placed in a metabolism cage (Maryland Plastic, Inc., New York) and urine samples were collected every 6 hours for 24 hours and again at 48 hours. During the collection period the urine samples were cooled in an ice bath and after collection they were frozen until analysis.

After a sample from each time period was counted, the urine taken over the first 24 hours was pooled and analyzed by anion exchange chromatography. Potential metabolites were added for cochromatography.

Respiratory products were collected from a total of six mice at each dosage level. Air was drawn through the glass metabolism cage and into a series of four traps. The first trap contained 30 ml. of a saturated aqueous solution of dimedone, and the last three contained 20 ml. each of 2-methoxyethanol–ethanolamine (2 to 1, by volume) which trapped respired CO_2 quantitatively. The traps were changed every 12 hours for 48 hours and the radioactivity was estimated by scintillation counting. The presence of formaldehyde was investigated by addition of unlabeled formaldehyde to the first trap and recrystallization of the dimedone derivative to constant specific activity.

Estimation of Tissue Glutathione. Male white mice (25 to 30 grams) were dosed orally with either 335 mg. per kg. of Sumithion or 85 mg. per kg. of sumioxon in 0.25 ml. of olive oil. Timing of the dosage was varied as much as possible to avoid the effects of any possible diurnal variations in glutathione levels of the tissues and none were observed in the results. Controls were dosed with olive oil. The mice were sacrificed at intervals from 30 minutes to 12 hours after dosage and the liver and brain were removed and washed with saline. Six to nine replicate mice were used for each time period. The tissues were extracted with 10 times their weight of the ethanol–pH 5.5 phosphate buffer system of Johnson (1966a). The nonprotein sulfhydryl content of duplicate samples of each extract was determined with 5,5′-dithiobis-(2-nitrobenzoic acid) reagent at pH 8.0. Recovery of glutathione added to the tissue extracts was about 90% by this method.

RESULTS

Metabolism of Methyl Paraoxon by Liver Supernatant. The fate of the methyl group in methyl paraoxon was followed with the $^{14}CH_3$-labeled compound. Initially the methyl paraoxon was incubated with whole homogenates of mouse liver and a search made for volatile radioactive metabolites. Failure to find significant amounts of such compounds, although *O*-dealkylation was occurring, indicated that the methyl group was removed and bound in a nonvolatile form. Attention, therefore, was turned to the observation of Fukami and Shishido (1966) that glutathione can act as a cofactor for this type of reaction. The effect of glutathione on detoxication of methyl paraoxon was explored with mouse liver supernatants (Table I). The over-all degradative activity of the supernatant is indicated by the amount of radioactivity not extractable from the incubates by organic solvent. The results, which represent the means of three to nine replicates, show that the supernatant alone has definite detoxifying ability but that this is greatly increased in the presence of glutathione. Glutathione in the absence of the enzyme has no effect on degradation. With 3.3 m*M* methyl paraoxon, the amount of water-soluble metabolites produced is linearly related to the total glutathione concentration in the supernatant, allowing for the natural content of glutathione in the supernatant which assays at an average of 0.55 m*M*. By extrapolation of such a plot it was estimated that in the complete absence of glutathione, 11% of the substrate added to the supernatant would be converted to water-soluble materials. Since in the

Table I. Stimulatory Effect of Glutathione on Degradation of Methyl Paraoxon by Mouse Liver Supernatant

Incubation Mixture	Incubation Time, Hr.	Radioactivity in Water Phase (% ± s.d.) Methyl Paraoxon Concn. 10 m*M*	3.3 m*M*	0.33 m*M*
Buffer alone	2	—	6.8 (±2.2)	—
3.3 m*M* glutathione	2	4.9	6.8 (±1.9)	—
Supernatant alone	2	8.4	18.1 (±4.5)	26.0
Supernatant + 0.33 m*M* glutathione	2	—	25.3 (±5.1)	42.2
Supernatant + 1.0 m*M* glutathione	2	—	37.4 (±9.5)	—
Supernatant + 3.3 m*M* glutathione	2	35.2	77.6 (±12.2)	98.2
Supernatant + 3.3 m*M* glutathione	1	—	59.7 (———)	—
Supernatant + 3.3 m*M* glutathione	0.5	—	39.2 (———)	—

Table II. Effect of Potential Cofactors and Inhibitors on Degradation of Methyl Paraoxon by Liver Supernatant

Activator (3.3 m*M*)	Inhibitor	Radioactivity in Water Phase, %	Inhibition of Enzymatic Degradation, %
None	None	18.1	—
L-Cysteine	None	16.5	—
S-(*N*-ethylsuccinimido) -glutathione	None	20.9	—
None	*N*-Ethylmaleimide	4.6	100
Glutathione	None	77.6	—
Glutathione	Methyl iodide (3.3 m*M*)	25.0	74.3
Glutathione	Dimethyl methylphosphonate (3.3 m*M*)	58.2	27.4
Glutathione	*p*-Nitrophenol (1.0 m*M*)	61.2	23.2

absence of the tissue, only 6% of the substrate partitions into water, there is an indication that a second glutathione-independent detoxication enzyme is present in the supernatant. Subsequent evidence suggests that this enzyme is of the phosphotriesterase type which converts methyl paraoxon to dimethyl phosphate (DMP). The high efficiency of the glutathione-mediated reaction can be seen in the incubation of 0.33 m*M* methyl paraoxon with 3.3 m*M* glutathione. Within 2 hours the destruction of the methyl paraoxon is virtually complete. At the relatively high substrate concentration of 10 m*M* the addition of 3.3 m*M* gluthathione causes an almost stoichiometric rise in the loss of methyl paraoxon.

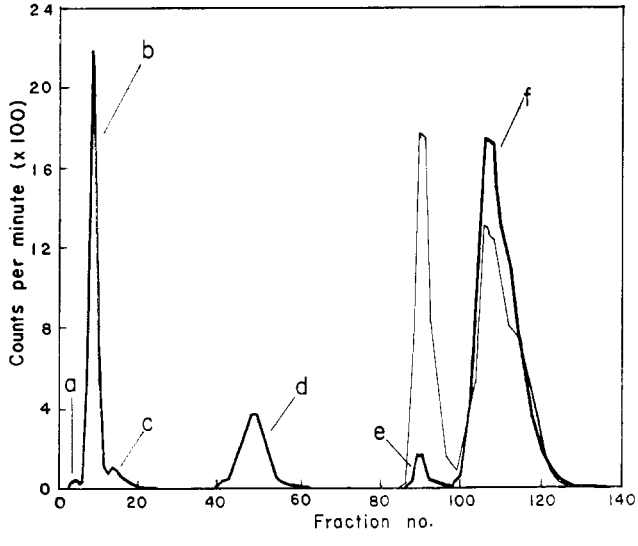

Figure 1. Anion exchange chromatogram of radioactive metabolites produced by mouse liver supernatant after incubation with 3.3 m*M* $^{14}CH_3$-labeled methyl paraoxon for 2 hours

Unlabeled methyl paraoxon (peak *e*) and demethyl methyl paraoxon (peak *f*) added for cochromatography. Peaks *a*, *b*, and *c* reduced in size to one fifth for more convenient presentation. Other peaks identified in text
—— Radioactivity
—— Nitrophenol

To understand further the properties of the glutathione-dependent system, other potential activators and inhibitors were examined (Table II). Clearly L-cysteine does not enhance the detoxication nor does *S*-(*N*-ethylsuccinimido) glutathione, in which the sulfhydryl group in glutathione is blocked. The sulfhydryl inhibitor *N*-ethylmaleimide added to the supernatant 5 minutes before the glutathione and methyl paraoxon completely prevented enzymatic degradation not only by *O*-dealkylation but by all other routes in addition. In the presence of added equimolar glutathione potential methyl donors such as methyl iodide and DMMP inhibit the glutathione effect.

The evidence above indicates that an enzymatic pathway of degradation of methyl paraoxon exists in the liver supernatant which has a specific requirement for glutathione and is inhibited by other methyl donors. The nature of this pathway was investigated by examining the individual metabolites produced by the supernatant. A typical separation on the anion exchange column in which methyl paraoxon and DES were added for cochromatography is shown in Figure 1. Six peaks of radioactivity were routinely observed in these separations, labeled *a* through *f* in the figure. Peaks *d*, *e*, and *f* were identified as DMP, methyl paraoxon, and DES, respectively. Peaks *a* and *c* were not identified, but the material in peak *c* gave a positive reaction to both ninhydrin and the platinic iodide reagent and was negative to the nitroprusside test. The identification of the radioactivity in peak *b* as predominantly *S*-methylglutathione is discussed subsequently.

The results of analysis of the water-soluble metabolites produced with varying concentrations of glutathione are

Table III. Influence of Glutathione Concentration on Pattern of Metabolism of Methyl Paraoxon by Liver Supernatants

Metabolite	3.3m*M* Methyl Paraoxon Added Glutathione			
	0	0.33 m*M*	1.0 m*M*	3.3 m*M*
S-Methylglutathione	19.3	35.9	38.5	46.6
Dimethyl phosphoric acid	63.8	25.0	21.7	4.1
Demethyl methyl paraoxon	16.0	38.7	39.2	48.2
Cleavage ratio: P-O-methyl / P-O-aryl	0.55	3.0	3.6	23

shown in Table III. The minor metabolites *a* and *c* have not been included. These results show that the effect of adding glutathione to the supernatant is specifically to increase the amount of DES and GSMe. Not only the proportion of DMP but the absolute amount produced decreases as the glutathione level rises. A further important point is that essentially equimolar amounts of GSMe and DES are produced at each concentration of glutathione. This observation supports the idea that the sulfhydryl of glutathione is a more or less direct acceptor of the methyl group removed from methyl paraoxon during dealkylation and that this is the only important mechanism of dealkylation in the supernatant fraction. It is not possible to say whether glutathione is alkylated directly by the methyl paraoxon or a more complex transalkylation sequence is involved.

Substrate Specificity of *O*-Dealkylase. Some preliminary evidence on the alkyl group specificity of the supernatant *O*-dealkylase was obtained with two series of organophosphates—i.e., the paraoxon family and the substituted vinyl phosphates. After incubation with the supernatant in the presence or absence of equimolar glutathione, the residual insecticide in the organic extract was estimated by gas chromatography. The results of duplicate determinations are seen in Table IV. Recoveries of unchanged insecticide considerably decreased on the addition of glutathione for all dimethyl phosphates (methyl paraoxon, sumioxon, and dichlorvos). On the other hand, the phosphates with higher alkyl groups (ethyl or isopropyl) show only a small enhancement of degradation on adding glutathione. Also, on comparing loss in buffer alone to loss with supernatant, only dichlorvos shows considerable metabolism by the supernatant in the absence of added glutathione.

Since the *O*-dealkylase accepts a variety of dimethyl phosphates and possibly other methyl donors such as methyl iodide and DMMP, it might have a general function in removing alkylating agents from the body. However, as Table IV shows, the relatively powerful alkylating agent TEPA showed little increase in degradation in the presence of glutathione. Thus, this *O*-dealkylase is probably not capable of efficiently degrading all types of alkylating agents.

Identification of Product of *O*-Dealkylation. The unknown radioactive metabolite *b* in Figure 1 was identified with some assurance by a series of chromatographic, electrophoretic, and chemical techniques. The fact that GSMe cochromatographs exactly with metabolite *b* on the anion exchange column is not convincing evidence, since all the acidic amino acids leave the column in approximately the same fractions. Chromatography of desalted samples of metabolite *b* on paper in systems B through E showed only one major radioactive spot (Table V). The R_f values for the standard compounds are the means of a series of determinations using ninhydrin for visualization. The values referred to as "metabolite" show the position of GSMe added for cochromatography along with the position of the radioactive peak on the same strip. The metabolite itself on these strips gave a positive response to ninhydrin and platinic iodide but not to nitroprusside and had the same R_f value whether GSMe was added or not. The results in Table V, taken from one of several replicate experiments, show that in every case the metabolite and GSMe behaved identically. A second, relatively minor, radioactive metabolite was present in the same eluate fractions as metabolite *b*, with an R_f in system B of 0.15. This compound was not identified but it gave a positive response to ninhydrin. It did not correspond to metabolite *a* or *c*.

The data in Table V also show that after acid hydrolysis of metabolite *b* the radioactive peak corresponding to GSMe is entirely lost and is replaced by a peak exactly corresponding to *S*-methyl-L-cysteine (CySMe). In addition, two other nonradioactive ninhydrin-positive compounds have appeared in approximately equal amounts which correspond to the other two amino acid constituents of GSMe, glycine and L-glutamate. The R_f values in the run represented in Table V are slightly lower than those for the standard compounds, possibly as a result of the acidic nature of the hydrolyzate.

The figures for electrophoretic mobility shown in Table V represent the same treatments as those for paper chromatography and confirm the identification of metabolite *b* as GSMe, yielding CySMe after hydrolysis.

Final proof that metabolite *b* is GSMe was obtained by reaction of the desalted eluate sample with 2,4-dinitrofluorobenzene to yield the DNP forms of the amino acids present. The ether extract of the acidified reaction mixture was subjected to two-dimensional thin-layer chromatography. As shown in Figure 2, the radioactivity ran as a single spot which exactly corresponded with the position of added DNP-GSMe.

Table IV. Effect of Glutathione on Degradation of Various Dialkyl Phosphates by Liver Supernatant

Substrate (3.3 m*M*)	Incubation Time, Hr.	% Recovery of Intact Substrate		
		Buffer alone	Supernatant alone	Supernatant plus 3.3 m*M* glutathione
Methyl paraoxon	2	93.5	81.4	23.3
Ethyl paraoxon	2	92.0	87.5	80.0
Isopropyl paraoxon	2	88.0	78.2	73.4
Sumioxon	2	95.4	79.2	26.6
Dichlorvos	2	74.0	12.5	0
Dichlorvos	1	81.5	56.5	8.6
Ethyl dichlorvos	1	85.3	78.8	72.8
Chlorfenvinphos (SD 7859)	1	91.9	82.8	81.6
TEPA	1/2	83.4	77.5	75.9

Table V. Identification of Metabolite *b* from Ion Exchange Column

Chromatography ($R_f \times 100$)	Amino Acids			
	L-Glu	Gly	GSMe	CySMe
System B				
Standards	28	33	57	72
Metabolite	—	—	58(58)[a]	—
Hydrolyzed	24	30	—	73(73)[a]
System C				
Standards	10	21	19	38
Metabolite	—	—	20(20)[a]	—
Hydrolyzed	7	18	—	36(36)[a]
System D				
Standards	49	57	59	78
Metabolite	—	—	55(55)[a]	—
Hydrolyzed	45	57	—	76(76)[a]
System E				
Standards	24	14	28	39
Metabolite	—	—	27(27)[a]	—
Electrophoresis (mobility, cm./4 hr.)				
pH 3.6				
Standards	+ 2.9	− 0.5	+ 5.4	+ 0.1
Metabolite	—	—	+ 5.4(+5.4)[a]	—
Hydrolyzed	+ 3.2	− 0.2	—	+ 0.4(+0.4)[a]
pH 3.0				
Standards	− 3.1	—	− 0.9	− 4.0
Metabolite	—	—	− 1.0(−1.0)[a]	—

[a] Position of radioactive peak on same strip.

Metabolism of Methyl Paraoxon by Other Subcellular Fractions. Since the oxidative *O*-dealkylation of xenobiotics is a well-established microsomal capability, such a reaction might be a second mechanism by which methyl paraoxon could be converted to DES. The results of incubations of methyl paraoxon with microsomal and mitochondrial preparations from mouse liver are shown in Table VI. As indicated by the amounts of water-soluble material produced, some degradation of methyl paraoxon is brought about by both these fractions, but unlike the supernatant, in neither case does glutathione have any activating effect and a consistent reduction in degradation was found with mitochondria in the presence of glutathione. No marked stimulation of detoxication was seen when the microsomal incubations were carried out in the presence of NADH or NADPH. Inclusion of nicotinamide and Mg^{2+} in the reaction mixture with NADPH did not increase the rate of metabolism of methyl paraoxon.

Ion exchange separation of the water-soluble metabolites produced by the microsomes showed only one metabolite, DMP, in the absence of added NADPH, but in the presence of NADPH very small amounts of second metabolite, leaving the column at the same place as DES, were observed. However, this contributed only 2.8% of the total water-soluble metabolites after incubation with 3.3 m*M* methyl paraoxon for 2 hours—i.e., less than 1% of the total substrate was converted to DES.

Since the general product of microsomal *O*-demethylation is formaldehyde, several attempts were made to identify this compound in the incubation mixture either by the chromotropic acid method of McFadyen (1945) or by the more sensitive reaction of radioactive formaldehyde with dimedone reagent (Donninger *et al.*, 1966). In the latter method, after the addition of carrier formaldehyde to the deproteinized microsomal reaction mixture and distillation of the acidified mixture into a dimedone solution, the formaldehyde adduct was recrystallized to constant specific activity. Neither method revealed any significant amount of formaldehyde, although semicarbazide was added in some experiments to trap any formaldehyde released.

The failure to detect more than traces of formaldehyde and the production of only insignificant amounts of the metabolite tentatively identified as DES raised the question of whether the *O*-dealkylating activity toward methyl paraoxon was naturally very low in these microsomal preparations or whether the microsomes, for some reason, had lost their activity during preparation. Measurements were made of the ability of some of the same microsomal preparations to *O*-dealkylate *p*-nitroanisole to *p*-nitrophenol (Kato and Gillette, 1965) in an analogous reaction to *O*-dealkylation of methyl paraoxon. In the presence of 1.3 m*M* NADPH and 0.33 m*M* substrate at 37° C. a linear rate of production of *p*-nitrophenol was observed for at least 10 minutes with an average rate of 38 nmoles

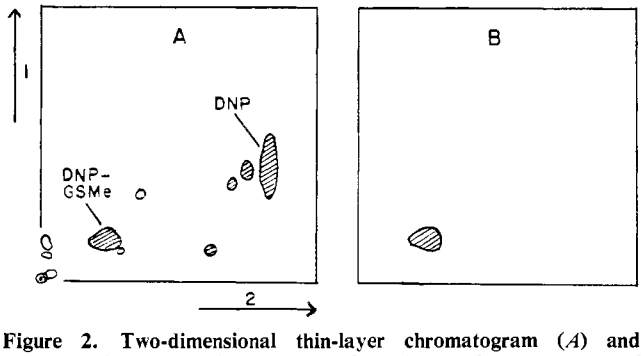

Figure 2. Two-dimensional thin-layer chromatogram (*A*) and corresponding autoradiogram (*B*) of products after treatment of ion exchange eluate containing metabolite *b* with 2,4-dinitrofluorobenzene

Developing solvents.
1. 1-Propanol–concentrated NH_4OH (4 to 1)
2. Chloroform–ethyl acetate–formic acid (9:9:2)

DNP-GSMe = 2,4-dinitrophenyl derivative of *S*-methylglutathione
DNP = 2,4-dinitrophenol

Table VI. Degradation of Methyl Paraoxon by Microsomes and Mitochondria of Mouse Liver and Effect of Cofactors

Incubation Mixture	Substrate (mM) 0.33			1.0	3.3
	Incubation Time, Hr. 1/2	1	2	1	2
	% Radioactivity in Water Phase				
Buffer alone	4.2	5.8	6.2	5.0	5.9
Microsomes alone	15.1	26.8	39.2	22.2	31.2
Microsomes + glutathione (3.3 mM)	—	—	40.1	—	32.7
Microsomes + NADH (2 mM)	—	—	39.6	—	30.1
Microsomes + NADPH (2 mM)	—	—	37.9	25.7	33.3
Mitochondria alone	—	—	—	—	12.4
Mitochondria + glutathione (3.3 mM)	—	—	—	—	8.4

per minute per gram of liver. This is a relatively high rate in comparison with literature values for rat liver microsomes and indicates that the microsomal *O*-dealkylating capacity of these preparations was not seriously impaired.

Metabolism in Vivo. The extent and nature of metabolism of methyl paraoxon were investigated in vivo for comparison with the observations on the liver subfractions.

In Figure 3 the rate of excretion of radioactivity is shown after oral dosage of mice with either 1 or 4 mg. per kg. of methyl paraoxon. At 4 mg. per kg. transient symptoms of poisoning were seen but no deaths occurred. No obvious symptoms resulted at 1 mg. per kg. The usual rapid excretion of water-soluble detoxification products in the urine was seen. A considerable output of respiratory radioactivity was observed, particularly at the higher dosage. At least 85% of the total respiratory radioactivity was collected in the CO_2 traps and less than 2% of the remaining radioactivity was precipitated as the dimedone adduct. The remaining 5 to 10% of each dose was found in the feces and excretion was virtually complete after 48 hours.

The nature and amounts of the individual metabolites present in the pooled 24-hour urine as analyzed by anion exchange chromatography are shown in Table VII.

At both the dosage levels the predominant metabolite is DMP, with DES as a second major contributant. The amount of DES is proportionally larger at the higher dosage level. A small amount of methyl paraoxon is excreted unchanged. The major difference between the metabolites excreted in the urine and those produced by the supernatant is the complete absence of GSMe in the urine sample. Three small unidentified peaks leave the column early in the general region of GSMe, but none corresponds exactly with GSMe itself, although one or more may represent a further metabolite of GSMe.

In a second series of experiments it was reasoned that if the glutathione transferase mechanism of *O*-dealkylation was an important degradative pathway in vivo, a measurable loss of liver glutathione should be observed after administration of a dimethyl phosphate ester. To achieve a higher internal concentration of organophosphate and thus a greater potential loss of glutathione, methyl parathion and methyl paraoxon were replaced by the much less toxic but closely related compounds Sumithion and sumioxon, which have a very similar pattern of metabolism (Hollingworth *et al.*, 1967a). The effects of these insecticides administered orally at 335 mg. per kg. for Sumithion and 85 mg. per kg. for sumioxon are shown in Figure 4. Each point represents the mean of six to nine individual determinations. The assay used measures total nonprotein thiol of the tissues, but since this thiol is composed largely of glutathione, the assay has been taken to measure tissue glutathione in the following discussion. This figure clearly shows that a considerable depletion of glutathione was caused in the liver by both Sumithion and sumioxon. This effect was greatest in the time 2 to 8 hours after dosage

Table VII. Metabolites of Methyl Paraoxon Excreted in Urine within 24 Hours

Metabolite	Metabolism of Methyl Paraoxon in Vivo % of Urinary Metabolites 1 mg./kg.	4 mg./kg.
Unknown 1	0.1	2.4
Unknown 2	3.3	2.9
Unknown 3	2.8	3.9
Dimethyl phosphoric acid	77.2	54.1
Methyl paraoxon	1.5	3.4
Demethyl methyl paraoxon	15.1	33.3

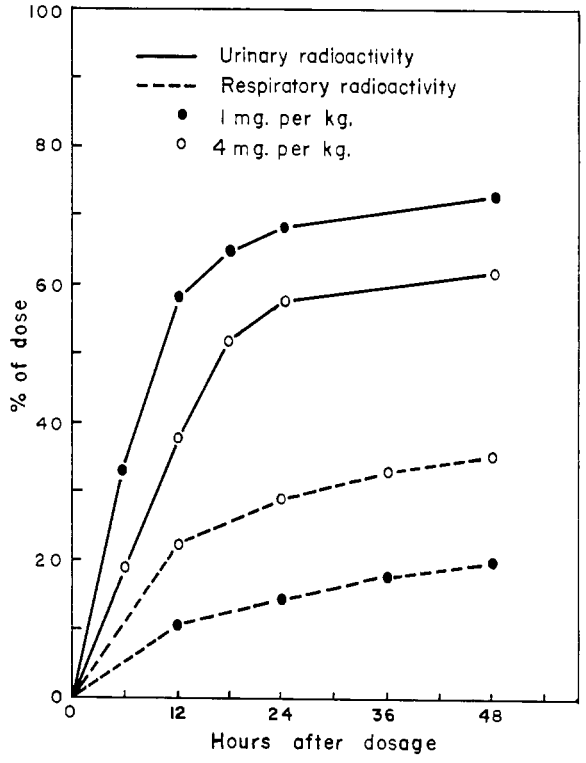

Figure 3. Rates of excretion of radioactivity in mice dosed orally with two levels of $^{14}CH_3$-labeled methyl paraoxon

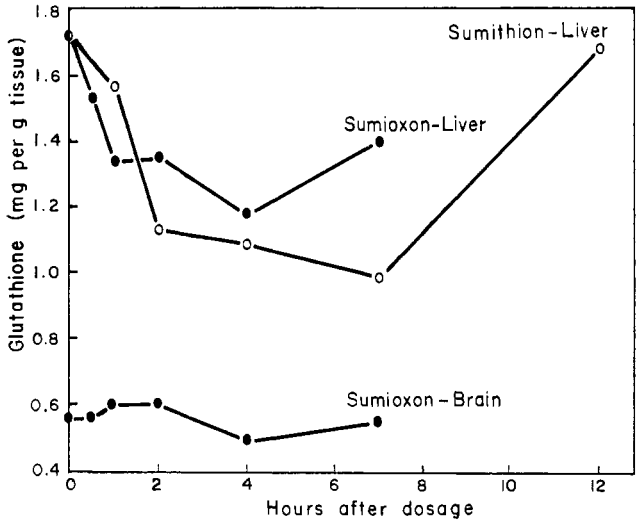

Figure 4. Depletion of glutathione in livers of mice dosed with 335 mg. per kg. of Sumithion and in livers and brains of mice dosed with 85 mg. per kg. of sumioxon

and within 12 hours of dosage the glutathione level had returned to normal. The 2-, 4-, and 7-hour liver glutathione levels with Sumithion were significantly different from the controls at the 0.001 probability level and the 1-, 2-, and 4-hour glutathione levels with sumioxon differed from the control at the 0.02 probability level.

Sumioxon was administered at a level close to that necessary to cause mortality and symptoms of poisoning were commonly observed. However, there was no correlation between the severity of poisoning and the extent of depletion of the liver glutathione in individual mice and in many cases animals with at most very mild symptomatology showed the lowest levels of glutathione. It is not likely that the observed depletion of glutathione is a direct result of poisoning. Sumithion at this dosage did not cause marked symptoms. No significant differences were found in the glutathione levels of the brains of the mice treated with sumioxon.

Further evidence of the importance of glutathione-mediated *O*-dealkylation in determining the mammalian toxicity of Sumithion was obtained by reducing the liver glutathione level in mice prior to dosage with the organophosphate. The mice were given 135 mg. per kg. of methyl iodide orally in 0.1 ml. of olive oil and then the required dose of Sumithion was administered either 1 or 12 hours later in 0.2 ml. of olive oil. Controls which did not receive methyl iodide received 0.1 ml. of oil. At 135 mg. per kg. methyl iodide caused no observable symptoms of poisoning and this dose is considerably below the LD_{50}. The results of these toxicity tests (Table VIII) clearly show a synergistic effect of methyl iodide when administered 1 hour but not 12 hours before the Sumithion. The effect was the same whether the methyl iodide was administered in the morning or in the evening. Assay of the liver glutathione levels of mice showed a decrease from 1.74 mg. per gram of liver in the controls to 0.62 mg. per gram in mice treated 1 hour previously with 135 mg. per kg. methyl iodide. Three hours after treatment the level was still strongly decreased at 0.58 mg. per gram but had returned to the original level within 12 hours.

Table VIII. Synergistic Effect of Short-Term Exposure to Methyl Iodide on Toxicity of Sumithion to Mice

Treatments, Mg. per Kg.		Interval between Treatments,	
Sumithion	Methyl iodide	Hours	Mortality
1260	—	—	LD_{50}
—	230	—	LD_{50}
1000	—	—	0/8
—	135	—	0/9
1000	135	1	9/9
750	135	1	7/8
1000	135	12	0/10

DISCUSSION

The products of *O*-dealkylation of methyl paraoxon in the liver supernatant are DES and GSMe. Recently Morello *et al.* (1967) and Hutson *et al.* (1968) also reported that GSMe is a product of dealkylation of various dimethyl phosphates in mammals, although in neither case was such a rigorous identification attempted. It is likely that a predominant mechanism of *O*-dealkylation of organophosphate insecticides in mammalian tissues in vitro is the transfer of an alkyl group to glutathione. A mechanism of this type is reasonable since methyl esters of strong acids have a marked alkylating capacity. Also it was observed in the present work that the *O*-dealkylase has a relatively low activity against ethyl and isopropyl phosphates and these esters generally have much weaker activity than their methyl analogs in alkylation reactions. Examples of clear specificity of attack against methyl rather than ethyl phosphate esters have been reported with a number of nucleophiles including pyridine (Nishizawa, 1961), triethylamine (Schrader, 1965), mercaptans (Pilgram, 1966), and iodide ion (Hollingworth, 1968).

In view of the importance of *O*-dealkylation as a detoxication mechanism of some organophosphates, particularly at dosage levels which are marginally toxic, as shown in the present work and by Hollingworth *et al.* (1967a), this relative ease of dealkylation may at least partially account for the observation that many organophosphate insecticides of low mammalian toxicity are dimethyl rather than diethyl esters. In some cases a dramatic increase in mammalian toxicity is seen on passing from the dimethyl to diethyl ester, as is the case with Sumithion (Nishizawa, 1961). No doubt other differences such as relative stability of the respective dialkyl phosphorylated cholinesterases may also contribute to the differential toxicity of alkyl phosphates.

There are several reports of the production of *S*-alkylglutathiones by mammalian tissue preparations after addition of various alkylating agents including methyl iodide (Johnson, 1966a, b) and ethylene dibromide (Nachtoni, 1966). There are some interesting similarities between the enzyme concerned in methyl iodide degradation as reported by Johnson and that mediating dealkylation of methyl paraoxon. Both are relatively specific for methyl transfers, under similar conditions they are inhibited to a similar extent by *p*-nitrophenol (Table II), and the reaction with methyl paraoxon is strongly inhibited by equimolar methyl iodide.

The fact that methyl paraoxon and other dimethyl phosphates alkylate glutathione in vivo raises the question of whether other, more critical, sites in the cell are also alkylated. Such reactions could conceivably contribute to some aspects of organophosphate symptomatology which are not immediately explicable by the phosphorylation of cholinesterase such as the teratogenic effects observed in birds' eggs (Dunachie and Fletcher, 1966) and other cytotoxic actions. Alkylation of various essential functional groups of serum enzymes by the dimethyl phosphonate insecticide trichlorfon has been observed by Dedek and Schwartz (1966).

Despite the high activity of the supernatant *O*-dealkylase in the presence of added glutathione, at the normal level of liver glutathione the major metabolite is DMP (64%) and not DES and GSMe (35%). However, dealkylation under in vitro conditions is probably disfavored by the oxidative and alkylative loss of glutathione which, in vivo, may be replaced continually. The in vitro product of microsomal detoxication and probably mitochondrial detoxication also is largely or wholly DMP. Thus, despite the unknown tissue concentrations of methyl paraoxon after oral dosage, it is reasonable that DMP is also the major metabolite produced in vivo (Table VII).

Turning to the microsomal metabolism, virtually all detoxication is accomplished by a mechanism which cleaves methyl paraoxon to DMP and *p*-nitrophenol and is NADPH-independent. This enzyme, which is currently being investigated, is of the phosphotriesterase type found in serum, liver, and many other tissues of mammals (Aldridge, 1953; Main, 1960).

The oxidative *O*-dealkylating capacity of the mouse liver microsomes was slight, in striking contrast to the rapid dealkylation reported with some diethyl phosphates by Donninger *et al.* (1966) using rabbit liver microsomes. Whether this disparity is a result of the different species, different substrates, or both is not yet clear.

The fact that two distinct mechanisms of *O*-dealkylation have been described for organophosphate esters in liver subfractions raises the question as to which may have been effective in vivo. In the present work the evidence from in vitro studies strongly favors the supernatant enzyme. However, the nonphysiological nature of the fractionation procedures, the relatively high concentrations of both substrate and added cofactors, and the fact that only the liver has been examined make extrapolation of in vitro observation to the living animal hazardous. Furthermore, the absence of identifiable amounts of GSMe, or even large amounts of unidentified compounds as possible metabolites of GSMe in the urine, may be taken as evidence that the microsomal, or some alternative system, is the source of the DES in the living animal. However, an alternative explanation is more likely.

The loss of glutathione from livers of mice dosed with Sumithion and sumioxon and the synergism of the toxicity of Sumithion by methyl iodide suggest that the alkylation of glutathione is, after all, an important mechanism in vivo. The maximum depletion of liver glutathione by sumioxon occurs 4 hours after dosage and amounts to 0.50 mg. of glutathione per gram of liver, equivalent to the dealkylation of 0.75 mg. of sumioxon in the liver or 30% of the total dose. The loss of glutathione is maximum 7 hours after treatment with Sumithion and is greater than that with sumioxon but amounts to only about 15% of the dose. However, these figures are probably considerable underestimates of the total dealkylation in the body by methyl transfer to glutathione particularly with Sumithion, since resynthesis of glutathione may be extremely rapid in the vertebrate liver (Kalser and Beck, 1963) and tissues other than the liver presumably are also capable of conducting this reaction. The brain, which has a comparatively low ability to degrade related organophosphates (Fukami and Shishido, 1966), shows no measurable loss of glutathione after administration of sumioxon. The interpretation of the synergistic activity of methyl iodide as a result of lowered liver glutathione is supported by the correlation between the glutathione level and the synergistic effectiveness. However, the alkylation of sites other than glutathione by the methyl iodide might contribute to the enhanced toxicity.

On the basis of this combined evidence it seems that glutathione alkylation is the major route of dealkylation in vivo and that the liver is responsible for a large part of the total dealkylation by the body under these conditions.

The redistribution and rate of resynthesis of glutathione in the liver may have a considerable influence on the rate of detoxication of relatively large doses of organophosphates, since the total liver content of glutathione is sufficient to account for the complete degradation of less than 100 mg. per kg. dosage of Sumithion and yet in previous work (Hollingworth *et al.*, 1967b) 45% of a dose of 850 mg. of Sumithion per kg. was excreted as desmethyl compounds within 24 hours. However, injections of glutathione into mice poisoned with Sumithion had no protective effect in preliminary experiments.

The question remains why no GSMe is found in the urine of treated mice. The probable reason is that GSMe is rapidly degraded in the body. A similarly surprising lack of GSMe in the urine of rats dosed with methyl iodide was reported by Johnson (1966a), who found that GSMe is stable in liver preparations but undergoes rapid degradation to CySMe and then to mercapturic acid by kidney homogenates. Further metabolism to volatile products such as carbon dioxide or thiols is likely (Johnson, 1966a).

The supposition that the methyl moiety used in alkylating glutathione is converted almost quantitatively to respirable products is supported by the excellent agreement between the amount of DES excreted in the urine in 24 hours and the amount of respiratory radioactivity in the same period. These figures are 15.1% of the dose as DES at 1 mg. per kg. and 33.3% at 4 mg. per kg. compared with 14.4 and 28.7%, respectively, as respiratory products at these dosages. Hassan and Zayed (1965) have similarly observed that about 25% of a dose of $^{14}CH_3$-labeled trichlorfon administered to rats at 200 mg. per kg. was recovered as respired CO_2 within 12 hours.

The conclusion is reached that both in vitro and in vivo the alkylation of glutathione constitutes the major pathway in mice by which methyl paraoxon and related dimethyl phosphates are dealkylated. This may not be valid for other animals or for other groups of organophosphates, particularly diethyl phosphate esters, which are much less active as alkylating agents.

NOMENCLATURE

CySMe = *S*-methyl-L-cysteine
DES = methyl *p*-nitrophenyl phosphate (demethyl methyl paraoxon)
DMCS = dimethyldichlorosilane
DMMP = dimethyl methylphosphonate
DMP = dimethyl phosphate
DNP- = dinitrophenyl-
GSMe = *S*-methylglutathione
TEPA = tris(1-aziridinyl)phosphine oxide

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