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STUDIES OF THE BINDING OF SULFUR RELEASED IN THE MIXED-FUNCTION OXIDASE-CATALYZED METABOLISM OF DIETHYL *p*-NITROPHENYL PHOSPHOROTHIONATE(PARATHION) TO DIETHYL *p*-NITROPHENYL PHOSPHATE (PARAOXON)*

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Abstract—Incubation of [^{35}S]labeled parathion with rat liver microsomes in the presence of an NADPH-generating system leads to the covalent binding of sulfur to the macromolecules of the microsomal membrane. The maximal binding of sulfur to microsomes requires the presence of NADPH, is increased using microsomes from phenobarbital and 3-methylcholanthrene-treated animals, and is inhibited by carbon monoxide. The majority, if not all, of the sulfur bound is in a form free of the remainder of the parathion molecule. These findings, coupled with the fact that the apparent K_m and V_{max} for sulfur binding are not statistically different from the apparent K_m and V_{max} for metabolism of parathion to paraoxon, indicate the sulfur bound is that released in the mixed-function oxidase-catalyzed metabolism of parathion to paraoxon. Under the conditions of the experiments described in this report, the binding of sulfur to microsomes decreases the concentration of cytochrome P-450 in the microsomes and inhibits slightly the rate of the mixed-function oxidase-catalyzed metabolism of benzphetamine.

WHEN *O*, *O*-diethyl *p*-nitrophenyl phosphorothionate (parathion) is incubated with microsomes isolated from the livers of rats and rabbits, a significant amount of sulfur becomes covalently bound to the macromolecules of the microsomes.[1,2] Further studies[2] have shown that the sulfur bound is in a form free of any phosphorus-containing residue of parathion.

The purpose of the studies described in this report was to examine if the sulfur bound is that which is released in the hepatic mixed-function oxidase-catalyzed metabolism of parathion to *O*, *O*-diethyl *p*-nitrophenyl phosphate (paraoxon). The metabolic significance of the binding of sulfur to the microsomal membrane was also examined.

METHODS

The ethyl-^{14}C and [^{35}S] parathion were products of the Amersham–Searle Corp. The benzphetamine was a generous gift of the Upjohn Co. The NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were products of Boehringer-

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Mannheim. Paraoxon was synthesized by coupling *O*, *O*-diethyl phosphorochloridate with sodium *p*-nitrophenylate according to the method of Neal.[3]

Adult male Sprague–Dawley rats were used in these studies. Liver microsomes were obtained by the Ca^{2+}-aggregation method[4] as modified by Cinti *et al.*[5] Studies of the metabolism of [^{14}C] parathion, [^{35}S] parathion or benzphetamine were carried out in 30-ml beakers using 2-3 mg microsomal protein/ml in 0·05 M Hepes (Sigma Chemical Co.) buffer (pH 7·8) in a total volume of 2 ml. NADPH was provided by the addition of a generating system consisting of 5 μmoles NADP, 15 μmoles glucose 6-phosphate and 1 unit of glucose 6-phosphate dehydrogenase. The mixture, was incubated for 15 min at 37° at a shaking rate of 200 rev/min in a gyroaction shaker. Binding of [^{35}S] or [^{14}C] labeled metabolites of parathion to microsomes was measured by first precipitating the microsomes from the incubation mixture by adding 0·1 ml of either 1 N HCl or $CaCl_2$ to a final concentration of 8·0 mM. The microsomes were isolated by centrifugation and washed overnight in 20·0 ml of diethyl ether containing 5·0 mM unlabeled parathion. The microsomes were washed for two additional 1-hr periods with fresh solutions of unlabeled parathion in diethyl ether. After this, the microsomes were solubilized in 1·0 ml of 1 N NaOH at 45–50°. After solubilization, an appropriate aliquot was removed for protein determination by the biuret method.[6] Water was added to the remaining solution so that the volume of the water plus NaOH was 3·5 ml and 11·5 ml of Aquasol (New England Nuclear Corp.) scintillation fluid was added. Shaking of this mixture resulted in a gel which could be counted efficiently in a liquid scintillation counter. An internal standard was used to correct for quench.

The rate of [^{14}C] paraoxon formation was determined by quantitating the amount of paraoxon and diethyl phosphate, the esterase-catalyzed product of paraoxon, in the incubation media and adding to this the amount of ^{14}C bound to the microsomes. In this determination, microsomes were precipitated from the incubation and separated by centrifugation, after which 1·0 mg of unlabeled paraoxon and 0·5 mg of unlabeled diethyl phosphorothionate, the other major mixed-function oxidase-catalyzed metabolite of parathion,[1,3] were added to each of the incubation supernatants. An aliquot of each supernatant was spotted on plastic Silica gel-coated thin-layer plates. These plates were then developed to a height of 15·0 cm using the solvent system hexane–chloroform–methanol (70:20:10, v/v). In this solvent system, parathion has an R_f of 1·0 and paraoxon an R_f of approximately 0·7, whereas diethyl phosphorothionate and diethyl phosphate remain at the origin. The plate was removed from the developing tank, dried, and the position of the paraoxon visualized using an ultraviolet light. The position of the paraoxon on the plate was marked with a pencil and the same plate was introduced into a developing tank containing the solvent system chloroform–methanol–aqueous 10% NH_3 (65:35:3·5, v/v). This solvent was allowed to migrate to a position just below the position of paraoxon on the plate. The plate was dried and sprayed with 0·5% $PdCl_2$ in 0·05 N HCl in order to visualize the location of diethyl phosphorothionate. Previous experience using radioautography of large numbers of these thin-layer plates had shown that diethyl phosphate trails the diethyl phosphorothionate in this solvent system by about 1·0 cm. The R_f values of diethyl phosphorothionate and diethyl phosphate in this solvent system are approximately 0·4 and 0·3 respectively. Using scissors, the portions of the plates containing paraoxon were cut from the plate and placed in scintillation

vials. Likewise, the portions of the plates starting 1 cm above the origin and including an area to just below the position of diethyl phosphorothionate were cut from the plates and placed in scintillation vials. These areas contained the diethyl phosphate. No metabolites other than diethyl phosphate have ever been detected in this area of the plate using radioautography. Ten ml of scintillation fluid was added to the vials and the radioactivity in these portions of the plates was determined by scintillation counting. The efficiency of counting of these metabolites was determined by spotting [^{14}C] parathion of known specific activity on pieces of the thin-layer plates approximately equal in size to those containing the metabolites and determining the scintillation counting efficiency.

The method used to measure the *N*-demethylation of benzphetamine has been described previously.[7] Cytochrome P-450 was measured by the method of Omura and Sato.[8]

Stoppered 50-ml Erlenmeyer flasks were used in experiments in which the effect of carbon monoxide on the rate of binding of sulfur to microsomes, the rates of formation of paraoxon and rates of metabolism of benzphetamine were compared. In these experiments, flasks containing microsomes and the NADPH-generating system in Hepes buffer were held on ice and the flasks evacuated using a high volume vacuum pump. The vacuum was then relieved with air or with carbon monoxide which had been passed through two deoxygenating gassing towers containing 200 ml of 0·09% sodium anthroquinone-2-sulfonate and 5% sodium dithionite in 0·1 N NaOH. This procedure was repeated three additional times. The flasks were then taken from the ice bath, placed in a gyroaction water bath and, after a 5-min temperature equilibration period, the reactions were started by injection of either labeled parathion or benzphetamine through the stopper.

The estimates of the apparent K_m and V_{max} values and the standard deviations of these estimates were calculated using a computer program written for that purpose.[9] The standard normal deviate test was used to test for significant differences between the apparent K_m and V_{max} values. All other statistical comparisons were made using the Student *t*-test.

RESULTS

Initial experiments dealing with the choice of solvent and washing procedures to be used in removing unmetabolized parathion and its unbound metabolites from microsomes led to the selection of diethyl ether as the solvent. The method chosen for washing the microsomes is indicated in Methods. Additional washes with diethyl ether containing unlabeled parathion beyond those described in Methods did not significantly reduce the amount of radioactivity bound to the microsomes. In addition, if a suspension of these ether–parathion washed microsomes was streaked at the bottom of a thin-layer plate coated with Silica gel G and the plate eluted with solvents which were known to migrate parathion and its metabolites from the origin, no radioactivity other than that at the origin could be detected by radioautography. The results of these preliminary experiments indicated that the methods used were capable of washing the microsomes essentially free of unmetabolized parathion and its metabolites.

The effect of the presence or absence of an NADPH-generating system on the amount of sulfur bound to rat liver microsomes after incubation with [^{35}S] labeled

TABLE 1. EFFECT OF THE ABSENCE OF AN NADPH-GENERATING SYSTEM ON ^{35}S BINDING TO RAT LIVER MICROSOMES*

NADPH-generating system	^{35}S bound† (nmoles/mg protein/15 min)
+	4·97 ± 0·03
−	0·07 ± 0·01

* Microsomes isolated from the pooled livers of three adult male Sprague–Dawley rats were incubated in triplicate with 1×10^{-4} M [^{35}S] parathion in the presence and absence of an NADPH-generating system. The nmoles of ^{35}S bound to the microsomes were calculated on the basis of the specific activity of the [^{35}S] parathion used in the incubations.

† Means ± standard deviations of the means of three determinations.

parathion is shown in Table 1. The amount of sulfur bound in the absence of an NADPH-generating system was approximately 2·0 per cent of that bound in the presence of a generating system. The small amount of radioactivity bound to the microsomes in the incubation not containing an NADPH-generating system may be due to the incomplete extraction of labeled parathion or its metabolites or a small residual activity of the mixed-function oxidase enzyme system in the absence of the exogenous NADPH-generating system. It is felt that this small amount of radioactivity is in fact due to a residual activity since, as we have previously indicated, additional washes with ether-parathion did not significantly reduce the amount of radioactivity bound to microsomes. Even if the radioactivity results from incomplete extraction of labeled parathion and its metabolites, the amount is so little as to not materially affect the accuracy of measurement of radioactivity bound when parathion is incubated with microsomes in the presence of an NADPH-generating system. This marked decrease in the amount of sulfur bound to the microsomes in the

TABLE 2. EFFECT OF CARBON MONOXIDE ON ^{35}S BINDING, PARAOXON FORMATION AND BENZPHETAMINE METABOLISM BY RAT LIVER MICROSOMES*

	Air	CO	Inhibition by CO (%)
^{35}S binding (nmoles ^{35}S bound/ 15 min/mg protein)†	4·95 ± 0·06	0·34 ± 0·04	93
Paraoxon formation (nmoles paraoxon formed/ 15 min/mg protein)†	5·29 ± 0·40	1·23 ± 0·21	77
Benzphetamine metabolism (nmoles HCHO formed/ 15 min/mg protein)†	62·78 ± 7·57	9·29 ± 1·99	85

* Microsomes isolated from the pooled livers of three adult male Sprague–Dawley rats were incubated in duplicate with 1×10^{-4} M [^{35}S] parathion, 1×10^{-4} M [^{14}C] parathion or 5×10^{-3} M benzphetamine in an atmosphere of air or carbon monoxide.

† Means ± standard deviations of the means of two determinations with each substrate. The values for paraoxon formation represent the sum of paraoxon and diethyl phosphate present in the reaction mixture at the end of the incubation period plus the amount of ^{14}C bound to the microsomes.

absence of the exogenous NADPH-generating system provides strong evidence that the sulfur binding is a result of the mixed-function oxidase-catalyzed metabolism of parathion to paraoxon.

The effect of carbon monoxide on the binding of sulfur to microsomes is shown in Table 2. In this experiment the amount of sulfur bound to microsomes incubated with [^{35}S] parathion in an air atmosphere is compared with that seen in an incubation carried out in an atmosphere enriched with carbon monoxide. The effect of carbon monoxide on sulfur binding using [^{35}S] parathion was also compared with its effect on paraoxon formation using an equivalent amount of microsomes from the same preparation and [^{14}C] labeled parathion. In addition, the effect of carbon monoxide on benzphetamine metabolism using the same preparation of microsomes was examined. As can be seen, the presence of carbon monoxide inhibited the rate of sulfur binding, the rate of paraoxon formation and the rate of benzphetamine metabolism to a similar degree. Although in this experiment the degree of inhibition of sulfur binding appears to be greater than that for paraoxon formation, the results of additional experiments were variable and, in general, there does not appear to be a significant difference in the degree of inhibition of these two reactions by carbon monoxide.

The results of previous studies[2] and the experiments described in Tables 1 and 2 suggest that the sulfur bound to the macromolecules of the microsomes is that released in the metabolism of parathion to paraoxon. This was examined further in an experiment in which the same concentrations of either [^{14}C] or [^{35}S] parathion were incubated with equal amounts of the same preparation of hepatic microsomes and the rate of sulfur binding was compared with the rate of paraoxon formation. The results of these experiments are shown in Table 3. The rate of sulfur binding was somewhat less than the rate of paraoxon formation at each concentration of labeled parathion used. The rate of sulfur binding averaged 72 per cent of the rate of paraoxon formation at all concentrations of parathion used in this experiment. However, in other experiments, as much as 95 per cent of the paraoxon formed in a 15-min incubation was accounted for by bound sulfur. The reason for the variability in the rate of sulfur binding as compared to the rate of paraoxon formation in various experiments is not known at this time. A double reciprocal plot of the data in Table

TABLE 3. COMPARISON OF THE AMOUNT OF ^{35}S BOUND AND PARAOXON FORMED ON INCUBATION OF VARIOUS CONCENTRATIONS OF [^{35}S] AND [^{14}C] PARATHION WITH MICROSOMES ISOLATED FROM THE LIVERS OF ADULT MALE RATS*

[^{35}S] or [^{14}C] parathion concn (M)	^{35}S bound† (nmoles/15 min/mg protein)	Paraoxon formed† (nmoles/15 min/mg protein)	^{35}S bound/ paraoxon formed (nmoles)
$0{\cdot}85 \times 10^{-5}$	$0{\cdot}90 \pm 0{\cdot}07$	$1{\cdot}25 \pm 0{\cdot}03$‡	0·72
$1{\cdot}70 \times 10^{-5}$	$1{\cdot}63 \pm 0{\cdot}10$	$2{\cdot}52 \pm 0{\cdot}19$‡	0·65
$3{\cdot}40 \times 10^{-5}$	$2{\cdot}29 \pm 0{\cdot}00$	$3{\cdot}34 \pm 0{\cdot}28$‡	0·69
$6{\cdot}80 \times 10^{-5}$	$2{\cdot}87 \pm 0{\cdot}17$	$3{\cdot}65 \pm 0{\cdot}08$‡	0·79

* The various concentrations of [^{35}S] and [^{14}C] parathion were incubated in duplicate with microsomes isolated from the pooled livers of three adult male Sprague-Dawley rats.

† Means ± standard deviations of the means of two determinations at each substrate concentration.

‡ These values represent the sum of paraoxon and diethyl phosphate present in the reaction mixture at the end of the incubation period plus the amount of ^{14}C bound to the microsomes.

TABLE 4. K_m AND V_{max} VALUES FOR THE METABOLISM OF PARATHION TO PARAOXON USING ^{35}S BINDING OR PARAOXON FORMATION AS THE MEASURE OF THE VELOCITY OF THE REACTION*

Velocity measurement	K_m (10^{-5} M)†	V_{max}† (nmoles/15 min/mg protein)
^{35}S binding	2·62 ± 0·32	4·00 ± 0·21
Paraoxon formation	1·79 ± 0·43	4·90 ± 0·43

* A double reciprocal plot was made of the data shown in Table 3 and the apparent K_m and V_{max} values were calculated using a computer program written for this purpose (see Methods). The sample variations in the apparent K_m and V_{max} values were obtained using the method of Searle.[10] The standard normal deviate test was used to test for significant differences between the apparent K_m and V_{max} values.

† These values are the parameter estimates ± standard deviations of the estimates determined as described previously.[11]

3 was made and the apparent K_m and V_{max} for paraoxon formation and the analogous kinetic constants for sulfur binding were calculated. These kinetic constants are shown in Table 4. A statistical treatment of the values shown in Table 4 indicates that there is not a significant difference between the apparent K_m or V_{max} values for paraoxon formation and the analogous kinetic constants for sulfur binding. The results of previous studies[2] and the experiments described in Tables 1–4 thus indicate that the sulfur bound when parathion is incubated with rat liver microsomes is that released in the metabolism of parathion to paraoxon. The fact that the V_{max} values for sulfur binding and paraoxon formation are not significantly different indicates that the rate-limiting step in sulfur binding is probably one of the series of reactions leading to the release of the sulfur from parathion. Thus the sulfur released in this reaction must be a very reactive species which binds to tissue components at a rate equal to or faster than the rate at which it is released from the parathion.

In a previous study[2] it was shown that, when [^{32}P] labeled parathion was incubated with rat hepatic microsomes, a portion of the parathion molecule labeled with ^{32}P became bound to microsomes. Additional experiments* showed that little or no binding of ^{32}P or ^{14}C occurred when [^{32}P] or [^{14}C] parathion was incubated with rat hepatic microsomes in the absence of NADPH. This binding of ^{32}P or ^{14}C to the microsomes was therefore thought to result from the interaction of paraoxon formed from parathion with nucleophilic groups on the microsomes, leading to the formation of a diethyl phosphate derivative of these nucleophilic groups. Further evidence supporting this hypothesis was that basic hydrolysis of the microsomes containing bound ^{32}P released only [^{32}P] diethyl phosphate.[2] In addition, preincubation of microsomes with [^{32}P] paraoxon resulted in the binding of significant amounts of ^{32}P, whereas incubation with unlabeled paraoxon followed by incubation with [^{32}P] labeled paraoxon did not lead to a detectable binding of radioactivity to the microsomes. Consequently, it was concluded that the ^{32}P bound to the microsomes was not paraoxon itself. In addition, these previous studies[2] had shown that no radioactivity could be detected in microsomes incubated with either of the parathion metabolites, [^{32}P]-diethylphosphate or ^{32}P, [^{35}S] diethylphosphorothionate. The

* B. J. Norman and R. A. Neal, unpublished observation.

TABLE 5. AMOUNT OF ^{14}C BOUND ON INCUBATION OF [^{14}C]PARATHION WITH MICROSOMES ISOLATED FROM THE LIVERS OF ADULT MALE RATS*

[^{14}C] parathion concn (M)	^{14}C bound† (nmoles/15 min/mg protein)
$0{\cdot}85 \times 10^{-5}$	$0{\cdot}285 \pm 0{\cdot}025$
$1{\cdot}70 \times 10^{-5}$	$0{\cdot}372 \pm 0{\cdot}013$
$3{\cdot}40 \times 10^{-5}$	$0{\cdot}639 \pm 0{\cdot}016$
$6{\cdot}80 \times 10^{-5}$	$0{\cdot}856 \pm 0{\cdot}069$

* These data represent the amount of ^{14}C bound to the microsomes at the varying concentrations of [^{14}C] parathion used in the experiments described in Table 3. The nmoles of ^{14}C bound to the microsomes were calculated on the basis of the specific activity of the [^{14}C] parathion used in these experiments.

† Means ± standard deviations of the means of two determinations at each substrate concentration.

results of these studies indicate that in order to determine accurately the amount of paraoxon formed in an incubation of [^{14}C] or [^{32}P] labeled parathion with microsomes, it is necessary to measure not only the paraoxon and diethyl phosphate free in the incubation media, but also the amount of ^{14}C or ^{32}P bound to the microsomes.

Table 5 indicates the amount of ^{14}C bound to the microsomes from the experiment described in Table 3. A double reciprocal plot of the data shown in Table 5 indicated that the apparent K_m and V_{max} for ^{14}C binding to the microsomes was $3{\cdot}77 \pm 0{\cdot}76 \times 10^{-5}$ M and $1{\cdot}33 \pm 0{\cdot}13$ nmoles ^{14}C bound/15 min/mg of protein respect-

TABLE 6. EFFECT OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE ON ^{35}S BINDING TO RAT LIVER MICROSOMES*

Treatment	^{35}S bound† (nmoles/15 min/mg protein)
None	$5{\cdot}12 \pm 0{\cdot}63$
PB‡	$16{\cdot}55 \pm 0{\cdot}81$
3-MC§	$13{\cdot}02 \pm 1{\cdot}37$

* [^{35}S] parathion (1×10^{-4} M) was incubated with microsomes isolated from the pooled livers of three phenobarbital-pretreated, 3-methylcholanthrene-pretreated or untreated adult male Sprague–Dawley rats using the procedures described in Methods.

† Means ± standard deviations of the means of three determinations with the sample of microsomes isolated from the pooled livers of the phenobarbital-treated, 3-methylcholanthrene-treated or untreated animals.

‡ The phenobarbital-treated animals received 50 mg/kg of sodium phenobarbital (PB) in distilled water by intraperitoneal injection each day for 5 days. The animals were sacrificed 24 hr after the last injection.

§ The 3-methylcholanthrene-treated animals received 20 mg/kg of 3-methylcholanthrene (3-MC) in corn oil by intraperitoneal injection and the animals were sacrificed 72 hr after the injection.

TABLE 7. EFFECT OF SULFUR BINDING ON THE ACTIVITY OF THE HEPATIC MIXED-FUNCTION OXIDASE ENZYMES TOWARD BENZPHETAMINE*

Preincubation conditions Parathion	NADPH	HCHO formed† (nmoles/15 min/mg protein)
+	+	22·3 ± 1·2
+	−	25·5 ± 0·7
−	+	39·4 ± 0·9
−	−	42·0 ± 0·6

* Microsomes isolated from the pooled livers of three adult male Sprague-Dawley rats were preincubated for 10 min under the conditions described in the table. The parathion concentration in the preincubation media was 1×10^{-4} M. The NADPH was provided by the NADPH-generating system described in Methods. After preincubation the microsomes were sedimented, washed once with Hepes buffer (pH 7·8, 0·05 M) and resuspended in the incubation mixture described in Methods. Benzphetamine (5×10^{-3} M) was added and the incubation carried out for 15 min.

† Means ± standard deviations of the means of two determinations under each preincubation condition.

ively. Since the data for ^{14}C binding shown in Table 5 were included in the data for paraoxon formation shown in Table 3, it was not possible to compare statistically the apparent K_m value for ^{14}C binding with that for paraoxon formation. A statistical comparison of the K_m value for ^{14}C binding with that for sulfur binding (Table 4) indicated there was not a significant difference. The V_{max} value for ^{14}C binding was, however, statistically different from the V_{max} value for sulfur binding. This suggests that the rate-limiting step in ^{14}C binding is reaction of paraoxon with some nucleophilic group or groups on the microsomes rather than the rate of formation of paraoxon from parathion. The nature of the groups with which paraoxon reacts can only be a matter of speculation at this time, but are believed to be, at least in part, the serine hydroxyl group of serine-active center esterases present in the microsomes.

The effect of phenobarbital and 3-methylcholanthrene pretreatment of animals on the ability of microsomes from the livers of these animals to catalyze sulfur binding is shown in Table 6. Note that there is a marked increase in sulfur binding to microsomes from animals pretreated with phenobarbital and 3-methylcholanthrene as compared to microsomes from untreated animals. This increase in sulfur binding is in line with previous data showing a similar increase in paraoxon formation when parathion was incubated with microsomes isolated from the livers of phenobarbital- and benzpyrene-treated rats and compared with the rate of paraoxon formation using hepatic microsomes from untreated rats.[3]

It was of interest to determine if the binding of sulfur to microsomes affected the ability of the mixed-function oxidase enzymes to catalyze the metabolism of substrates for this enzyme system. In these experiments, microsomes from untreated animals were preincubated with parathion in the presence and absence of an NADPH-generating system. As shown in Table 1, incubation of parathion with microsomes in the absence of an NADPH-generating system leads to little binding of sulfur to the microsomes. After the preincubation, the microsomes were sedimented, washed with buffer by resuspension and centrifugation, and examined for their ability to carry out the *N*-demethylation of benzphetamine. Since incubation of microsomes

with an NADPH-generating system but no substrate had been reported to decrease the activity of the mixed-function oxidase enzyme system,[12] samples of this same microsomal preparation were also preincubated in the presence and absence of an NADPH-generating system but no parathion. The microsomes from these incubations were then sedimented, washed with buffer and their ability to catalyze the *N*-demethylation of benzphetamine examined. An examination of these data (Table 7) indicates that microsomes preincubated with parathion in the presence of an NADPH-generating system metabolized benzphetamine at a significantly ($P < 0.05$) slower rate than did microsomes preincubated with parathion in the absence of an NADPH-generating system. There was no significant difference in the rate of benzphetamine metabolism by microsomes preincubated with NADPH alone as compared to those preincubated in the absence of both NADPH and parathion. Microsomes preincubated with parathion metabolized benzphetamine at a considerably slower rate than those not preincubated with parathion. The most logical explanation for the difference in the ability of these microsomes to metabolize benzphetamine was the incomplete removal of parathion bound to the microsomes preincubated with this compound. This residual parathion could act as a competitive inhibitor of the metabolism of benzphetamine. Previous work in this laboratory has shown that parathion is a competitive inhibitor of benzphetamine metabolism.*

Since the rate of benzphetamine metabolism using microsomes preincubated with parathion in the presence of an NADPH-generating system was less than that seen with microsomes preincubated with parathion in the absence of an NADPH-generating system, the effect of incubation of microsomes with parathion in the presence and absence of an NADPH-generating system on the concentration of cytochrome P-450 in the microsomes was also examined. These data are shown in Table 8. The results of these experiments indicate that incubation of microsomes with parathion in the presence of an NADPH-generating system significantly ($P < 0.05$) decreases the concentration of cytochrome P-450 in the microsomes as compared to those incubated with parathion in the absence of an NADPH-generating system and those

TABLE 8. EFFECT OF SULFUR BINDING ON THE CONCENTRATION OF CYTOCHROME P-450 IN RAT LIVER MICROSOMES*

Incubation conditions		Cytochrome P-450†
Parathion	NADPH	(nmoles/mg protein)
+	+	0·342 ± 0·006
+	−	0·476 ± 0·020
−	+	0·438 ± 0·008
−	−	0·436 ± 0·009

* Microsomes isolated from the pooled livers of three adult male Sprague–Dawley rats were incubated for 10 min under the conditions described in the table. The parathion concentration in the incubation media was 1×10^{-4} M. The NADPH was provided by the NADPH-generating system described in Methods. After the incubation, the microsomes were sedimented, washed once with Hepes buffer, (pH 7·8, 0·05 M) and the concentration of cytochrome P-450 in the microsomes determined as described in Methods.

† Means ± standard deviations of the means of two determinations under each incubation condition.

* J. Roth and R. A. Neal, unpublished observations.

TABLE 9. EFFECT OF INCUBATION OF MICROSOMES WITH PARAOXON ON THE CONCENTRATION OF CYTOCHROME P-450 IN THE MICROSOMES*

Incubation condition	Cytochrome P-450† (nmoles/mg protein)
With paraoxon (1×10^{-5} M)	0·427 ± 0·005
Without paraoxon	0·432 ± 0·021

* Microsomes isolated from the pooled livers of three adult male Sprague–Dawley rats were incubated for 10 min in 2·0 ml Hepes buffer (pH 7·8, 0·05 M) with and without paraoxon. The incubation procedures are described in Methods. After the incubation, the microsomes were sedimented, washed once with the same Hepes buffer, and the concentration of cytochrome P-450 determined as described in Methods.

† Means ± standard deviations of the means of three incubations in the presence and in the absence of paraoxon.

incubated with and without an NADPH-generating system but no parathion. There was no significant difference in the concentration of cytochrome P-450 in the microsomes incubated with parathion in the absence of NADPH and those incubated with or without NADPH but no parathion.

The effect of incubation of microsomes with paraoxon on the concentration of cytochrome P-450 in the microsomes was also examined. The results of these experiments are shown in Table 9. It is possibe that the decrease in benzphetamine metabolism (Table 7) and the concentration of cytochrome P-450 (Table 8) seen when microsomes are incubated with parathion in the presence of an NADPH-generating system may have been the result of the reaction of sulfur, paraoxon, diethyl phosphate or diethyl phosphorothionate with cytochrome P-450. Previous studies[2] have shown that incubation of [^{32}P] paraoxon with microsomes leads to the binding of radioactivity. However, no binding of radioactivity was detected when microsomes were incubated with [^{32}P] diethyl phosphate and ^{32}P, [^{35}S] diethyl phosphorothionate. As can be seen in Table 9, incubation with paraoxon has no effect on the concentration of cytochrome P-450 in the microsomes. Therefore the slight decrease in enzyme activity and the greater decrease in the concentration of cytochrome P-450 seen on incubation of microsomes with parathion in the presence of an NADPH-generating system is most probably due to the binding of sulfur.

DISCUSSION

The results of these experiments and those previously published[2] indicate that sulfur bound to microsomes incubated with [^{35}S]labeled parathion in the presence of NADPH is that which is released during the mixed-function oxidase-catalyzed metabolism of parathion to paraoxon. A theoretical basis for the mixed-function oxidase-catalyzed release of sulfur from parathion and its binding to microsomes has recently been reported.[11,13]

The species of sulfur released in the metabolism of parathion to paraoxon appears to be highly reactive. This is evidenced by the fact that the V_{max} for sulfur binding is not statistically different from the V_{max} for paraoxon formation. It therefore appears that the release of the sulfur rather than its binding is the rate-limiting step in the

reaction. Atomic sufur, the chemical form of the sulfur proposed to be released in the mixed-function oxidase-catalyzed metabolism of parathion to paraoxon,[11,13] should have this highly reactive property.

In the experiments reported here, all of the sulfur released in the metabolism of parathion to paraoxon does not become bound to microsomes. This variance between sulfur binding and paraoxon formation could be accounted for in a number of ways. First, the sulfur may react with some component of the incubation mixture other than the microsomes. If the sulfur released is an electrophile, as has been proposed,[13] suitable candidates for the reactive molecules outside the microsomes are water, the amine groups on NADP or NADPH, the oxygen atoms of the phosphate groups of glucose 6-phosphate, NADP and NADPH, various nucleophiles on glucose 6-phosphate dehydrogenase or the buffer. Second, since the sulfur released becomes bound to all three of the major macromolecular species present in microsomes, namely proteins, nucleic acids and lipids,* some of the lipids to which sulfur is bound may be removed in the washing of the microsomes with diethyl ether. It is not clear at this time which, if any, of these alternatives is most important.

The results described in Tables 3 and 5 indicate that under the conditions of these experiments approximately 20 per cent of the paraoxon formed in the mixed-function oxidase-catalyzed metabolism of parathion become bound to the microsomes. This binding is thought to result from the reaction of paraoxon with a limited number of nucleophilic groups on the microsomes to form a diethyl phosphate derivative of these nucleophiles. Evidence supporting the view that there are a limited number of binding sites for paraoxon comes from an experiment in which both [^{32}P] parathion plus NADPH and [^{32}P] paraoxon were incubated separately with the same sample of rabbit liver microsomes.[2] The amount of [^{32}P] paraoxon formed from [^{32}P] parathion was approximately 0·14 μmole, yet the amount of ^{32}P bound was not significantly different from that seen in the presence of 0·69 μmole [^{32}P] paraoxon. The results of the experiments described in Tables 3 and 5 indicate that if one measures only the amount of paraoxon and diethyl phosphate in the reaction media after the incubation of parathion with microsomes *in vitro*, the amount of paraoxon formed in the reaction will be underestimated by approximately 20 per cent. In all probability, this caution should also apply to the measurement of the mixed-function oxidase-catalyzed metabolism of phosphorothionate insecticides other than parathion.

Concerning the use of [^{35}S] sulfur binding as a measure of the rate of metabolism of parathion to paraoxon, it appears that an underestimation of the rate of this reaction by approximately 25 per cent may be seen in some experiments. However, the per cent error may in fact be somewhat variable, since in one experiment we found that nearly all (95 per cent) of the sulfur released was bound to the microsomes. The major advantage of using sulfur binding as the measure of the rate of parathion to paraoxon metabolism by hepatic microsomes is the elimination of the tedious spotting of aliquots of the supernatants and the measurement of the amount of ^{14}C or ^{32}P from ethyl-[^{14}C] parathion or [^{32}P] parathion bound to the microsomes that is required to measure paraoxon formation accurately and directly.

It appears that the decrease in both the mixed-function oxidase activity and concentration of cytochrome P-450 seen on incubation of parathion with hepatic

* R. Crowder and R. A. Neal, unpublished observations.

microsomes in the presence of NADPH is due to the binding of sulfur released in the reaction. Theoretically, it is also possible that the reactive compound, paraoxon, could also be responsible for these decreases. However, the data in Table 9, showing no decrease in cytochrome P-450 when paraoxon is incubated with microsomes, tend to disprove this. Although other explanations are possible, it appears most likely that sulfur released in the metabolism of parathion to paraoxon binds to the heme ligands and/or the amino acid side chains involved in the catalytic mechanism of the reactions catalyzed by cytochrome P-450. The consequence of this binding of sulfur is a decrease in both the activity of the mixed-function oxidase enzyme system and the amount of cytochrome P-450 detectable as its carbon monoxide complex.

The degree of inhibition of mixed-function oxidase activity seen on incubation of microsomes with parathion and NADPH (Table 7) appears to be less than the decrease in concentration of cytochrome P-450 (Table 8). However, since these were separate experiments, it is not possible to draw any definite conclusions regarding the lack of correlation at this time.

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