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Metabolism of Diethyl *p*-Nitrophenyl Phosphorothionate (Parathion) by a Reconstituted Mixed-Function Oxidase Enzyme System: Studies of the Covalent Binding of the Sulfur Atom

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SUMMARY

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During the metabolism of parathion by a reconstituted mixed-function oxidase system from rat liver, the sulfur-containing portion of the molecule becomes covalently bound, predominantly, if not exclusively, to cytochrome P-450. Companion experiments using ^{14}C-labeled parathion indicated that more than 95% of the sulfur bound to cytochrome P-450 is free of the remainder of the parathion molecule. Thus the bound sulfur is the sulfur atom released in the metabolism of parathion to paraoxon. Investigations concerning the nature of the covalent bond between the sulfur atom and cytochrome P-450 suggested that about 50% becomes bound to the side chain of the cysteine in the cytochrome P-450 apoenzyme, forming a hydrodisulfide. Binding of the sulfur to cytochrome P-450 of the reconstituted system apparently leads to cross-linking of the proteins of the reconstituted system to form high molecular weight complexes. Examination of the binding of sulfur to the macromolecules of intact rat liver microsomes on incubation with parathion revealed that the majority of the atomic sulfur is bound to protein(s) precipitated by an antibody to rat liver cytochrome P-450. This binding of sulfur to the macromolecules of intact microsomes also leads to the formation of high molecular weight complexes as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

The administration of thiono sulfur-containing compounds to experimental animals *in vivo* (1-4) or incubation with hepatic microsomes *in vitro* (1, 3-7) leads to a decrease in the level of cytochrome P-450 detectable as its carbon monoxide complex and to a decrease in the rate of metabolism of substrates for the hepatic mixed-function oxidase system. In addition, centrilobular hepatic necrosis is seen when some of these compounds are administered *in vivo* (1-3).

It has recently been suggested that the inhibition of the mixed-function oxidase system and the decrease in the concentration of cytochrome P-450 seen on incuba-

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tion of rat liver microsomes with carbon disulfide are the results of covalent binding of the sulfur atom released in the mixed-function oxidase-catalyzed metabolism of CS_2 to carbonyl sulfide (3, 5, 8). The centrilobular hepatic necrosis seen on administration of CS_2 to phenobarbital-treated rats is also thought to result from the mixed-function oxidase-catalyzed metabolism of this compound to a reactive intermediate (2, 3, 5). The nature of the covalent bond formed between the sulfur atom released in the mixed-function oxidase-catalyzed metabolism of CS_2 and the macromolecules of the microsomes has recently been investigated (9). This study revealed that approximately 50% of the sulfur was bound in the form of a hydrodisulfide (R–S–S–H). This hydrodisulfide is most likely formed in a reaction between the sulfur atom released in the metabolism of CS_2 to COS and the side chain of the cysteine present in the proteins of the microsomes.

The administration of the phosphorothionate insecticide fenitrothion (dimethyl 3-methyl-4-nitrophenyl phosphorothionate) to mice *in vivo* results in a decrease in the activity of the hepatic mixed-function oxidase system(s) toward aniline and aminopyrine (4). The phosphorothionate insecticide parathion (diethyl *p*-nitrophenyl phosphorothionate) inhibits mixed-function oxidase activity and decreases the level of cytochrome P-450 detectable as its carbon monoxide complex when incubated with rat liver microsomes in the presence of NADPH (7). The results of the latter studies indicated that, like the results obtained using CS_2 (3, 5, 8), the inhibition of mixed-function oxidase activity and the decrease in cytochrome P-450 result from covalent binding of the sulfur atom released in the mixed-function oxidase-catalyzed metabolism of parathion to paraoxon (diethyl *p*-nitrophenyl phosphate).

In the present study we have examined the binding of the sulfur atom of parathion to the proteins of a reconstituted mixed-function oxidase system isolated from rat liver micorsomes. The major purpose of these studies was to determine whether the sulfur atom was bound to cytochrome P-450, NADPH-cytochrome *c* reductase, or both. In addition, we have examined the nature of the covalent linkage between the sulfur atom and the proteins of the reconstituted system.

MATERIALS AND METHODS

Preparation of microsomes. Male Sprague-Dawley rats weighing 150–200 g were given 0.1% sodium phenobarbital in their drinking water for 3 days. The animals were killed by decapitation, the livers were removed and perfused with 1.15% KCl, and the microsomes were prepared as described previously (10).

Purification of cytochrome P-450 and NADPH-cytochrome c reductase from phenobarbital-treated rats. Cytochrome P-450 was purified from the livers of phenobarbital-treated rats as described previously (11). The method used was a modification of that reported by Imai and Sato (12) for purification of rabbit liver cytochrome P-450. The specific content of cytochrome P-450 used in these studies was 15.4–16.4 nmoles/mg of protein. The specific activity of NADPH-cytochrome *c* reductase ranged from 20.5 to 28.3 units/mg of protein. This enzyme, which was purified as described previously (11), is 42–58% pure compared with the specific activity of homogeneous rat liver NADPH-cytochrome *c* reductase (13). SDS[2]-polyacrylamide gel electrophoresis of the reconstituted system or intact microsomes after incubation with parathion or other substrates was carried out using 7.5% gels and a buffer system consisting of 0.2 M Tris-acetate (pH 6.0) containing 0.1% (w/v) SDS. Prior to application to the gels, the samples were treated for 5 min at 100° with 20 mM Tris-acetate (pH 6.0) containing 2 M urea, 1% SDS, 0.002% bromphenol blue, and 1 mM EDTA. Protein bands were visualized by staining the gels with 0.2% Coomassie blue R-250 in methanol–acetic acid–water (5:1:5) for 30 min and destaining by diffusion overnight in a 10% acetic

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

acid solution containing 10% (v/v) methanol. In order to determine the distribution of ^{35}S bound to the proteins separated on SDS-gel electrophoresis, the gels were sliced at 1-mm intervals and each slice was dissolved by placing it in 1 ml of 15% hydrogen peroxide in a sealed 20-ml scintillation counting vial and heating at 70° for 16 hr. The radioactivity in the slices was determined by scintillation counting. Quenching was corrected for using an internal standard. Partially purified antibody from rabbits immunized against rat liver cytochrome P-450 was prepared as described previously (11).

Analytical methods. Protein was determined by the method of Lowry *et al.* (14). Cytochrome P-450 was determined by the method of Omura and Sato (15) in the presence of 20% glycerol and 0.2% Emulgen 913 (12). NADPH-cytochrome *c* reductase was assayed by measuring the NADPH-dependent reduction of cytochrome *c* (16). The spectrophotometric measurements were carried out at room temperature using a Cary 15 spectrophotometer. Studies of the metabolism of parathion were carried out as described previously (11).

Materials. NADPH and horse heart cytochrome *c* were obtained from Boehringer/Mannheim; Sephadex G-25, from Pharmacia; and SDS-polyacrylamide gel (7.5%) and Tris-acetate buffer containing SDS, from Bio-Rad. [^{35}S]- and [*ethyl*-^{14}C]Parathion were obtained from Amersham-Searle; these preparations had an average specific activity of 5 μCi/μmole and were more than 99% pure.

TABLE 1

Binding of sulfur atom of parathion to rat liver cytochrome P-450

The complete incubation mixture contained 0.5 nmole of cytochrome P-450, 0.25 unit of NADPH-cytochrome *c* reductase, 100 μg of dilauroyl-L-3-phosphatidylcholine, 0.05 M HEPES buffer (pH 7.8), 0.015 M $MgCl_2$, 100 μg of sodium deoxycholate, 0.1 mM EDTA, 0.1 mM [^{35}S]- or [*ethyl*-^{14}C]parathion, and 0.1 mM NADPH in a total volume of 1.0 ml. The reaction mixtures were incubated at 37° for 5 min. The reactions were stopped by placing the incubation in an ice-water bath. After approximately 5 min, 3 mg of partially purified antibody to cytochrome P-450 were added, and the mixtures were allowed to stand at 0-4° for 16 hr. Previous studies had shown that all of the cytochrome P-450 was precipitated under these conditions (11). The precipitate representing cytochrome P-450 combined with its antibody was separated by centrifugation at 2000 × *g* in the cold and washed successively with 5 ml each of 50 mM potassium phosphate, pH 7.25, containing 0.85% sodium chloride (twice), 100% ethanol containing 1 mM unlabeled parathion, and 100% ethanol. The precipitate was dried under a stream of nitrogen and dissolved in 1 ml of NaOH, and radioactivity was determined by scintillation counting. The data represent the means ± standard deviations of duplicate determinations.

Conditions	^{35}S or ^{14}C binding
	nmoles/nmole P-450/ 5 min
[^{35}S]Parathion	
Complete system	1.02 ± 0.02
−NADPH	0.04 ± 0.01
−NADPH-cytochrome *c* reductase	0.04 ± 0.00
−Dilauroylphosphatidylcholine	0.41 ± 0.00
−Deoxycholate	0.80 ± 0.01
$-Mg^{++}$	1.05 ± 0.02
[^{14}C]Parathion	
Complete system	0.020 ± 0.002

RESULTS

Table 1 shows the results of an examination of the binding of the sulfur atom of parathion to cytochrome P-450 of a reconstituted rat liver mixed-function oxidase system. The requirements, in terms of components of the reconstituted system, for covalent binding of sulfur are quite similar to those previously observed for the metabolism of parathion to paraoxon, diethylphosphorothioic acid, and diethylphosphoric acid (11). This result was not unexpected, since a previous study had established that the sulfur which covalently binds to microsomes incubated with [^{35}S]parathion is predominantly that sulfur atom which is released in the metabolism of parathion to paraoxon (7). When the incubation was carried out using [*ethyl*-^{14}C]parathion, only about 2% as much ^{14}C as ^{35}S was bound to cytochrome P-450 in the presence of the complete system. Therefore the majority of the sulfur bound to cytochrome P-450 under the con-

ditions described in Table 1 is free of the remainder of the parathion molecule.

Shown in Table 2 is the effect on cytochrome P-450 of incubating parathion with the reconstituted system under various conditions. Some apparent loss of cytochrome P-450 occurs in the absence of necessary cofactors or the substrate for the reconstituted system. This loss in cytochrome P-450 is not due to conversion to cytochrome P-420. Rather, it is most likely due to incomplete transfer of the small amount of enzyme (170 μg) from the incubation flasks to the cuvette used to measure cytochrome P-450, probably because of adsorption of cytochrome P-450 to the walls of the incubation flask. Under conditions in which the data in Table 1 show that maximum binding of sulfur occurs (complete system), about 45% of the cytochrome P-450 added to the incubation is no longer detectable as its carbon monoxide complex. If it is assumed that about 20% of this loss of cytochrome P-450 is due to incomplete transfer of the enzyme from the incubation flask to the cuvette used to measure cytochrome P-450, the actual loss due to the incubation is only about 25%. In contrast, under conditions in which the reconstituted system was inactive (NADPH and reductase omitted), or when parathion was not present, or when paraoxon, which is not a substrate for the reconstituted mixed-function oxidase enzyme system, was substituted for parathion in the complete system, only 14–24% of the original cytochrome P-450 was no longer detectable. These and previous data obtained using whole microsomes (7) indicate that the larger decrease in P-450 seen in the presence of the complete system is due to covalent binding of the sulfur atom released in the metabolism of parathion to paraoxon.

TABLE 2

Decrease in cytochrome P-450 content on incubation of parathion with rat liver reconstituted mixed-function oxidase system

Cytochrome P-450 (2.58 nmoles) was first incubated at 37° for 3 min in the presence of NADPH-cytochrome *c* reductase (0.5 unit), dilauroyl-L-3-phosphatidylcholine (30 μg), parathion (0.1 mM) or paraoxon (0.1 mM), sodium deoxycholate (100 μg), HEPES buffer (0.05 M, pH 7.8), EDTA (0.1 mM), and $MgCl_2$ (0.015 M). The reaction was started by the addition of an NADPH-generating system (NADP, 2.5 mM; glucose 6-phosphate, 7.5 mM; and glucose 6-phosphate dehydrogenase, 0.5 unit). The volume of the reaction mixture was 1 ml. After incubation for 5 min, Emulgen 913 (0.2%) and glycerol (20%) were added, the mixtures were transferred to a spectrophotometric cuvette, and the content of cytochrome P-450 was measured. The results represent the means ± standard deviations of duplicate determinations.

Conditions	Cytochrome P-450 remaining	
	nmoles	%
Complete system	1.44 ± 0.11	56
−NADPH	1.97 ± 0.00	76
−Reductase	2.10 ± 0.00	81
−Parathion	2.23 ± 0.04	86
−Parathion and paraoxon	2.06 ± 0.01	80

Using whole microsomes, 70–95% of the sulfur released in the mixed-function oxidase-catalyzed metabolism of parathion to paraoxon became bound to the macromolecules of the microsomes used in the incubation (7). In the experiment described in Table 1, only about 25% of the sulfur released became bound to cytochrome P-450. A number of factors may account for this difference. First, some of the P-450 is solubilized from the antibody-P-450 complex during the washing of the precipitate. Furthermore, the sulfur released by the reconstituted system may more readily escape the region of the active site of the cytochrome P-450 and react with low molecular weight nucleophiles, such as water, in the incubation medium. Alternatively, the sulfur released by the reconstituted system may bind not only to cytochrome P-450 but to NADPH-cytochrome *c* reductase as well. The latter possibility was examined in the experiment shown in Fig. 1. In this experiment the reconstituted system was incubated with [^{35}S]parathion, followed by dialysis of the reaction mixture to remove unmetabolized parathion, its noncovalently bound metabolites, and other low molecular weight radiolabeled compounds. Increasing amounts of partially purified antibody were then added to aliquots of this dialyzed reaction mixture, and the amount of radioactivity present in the P-

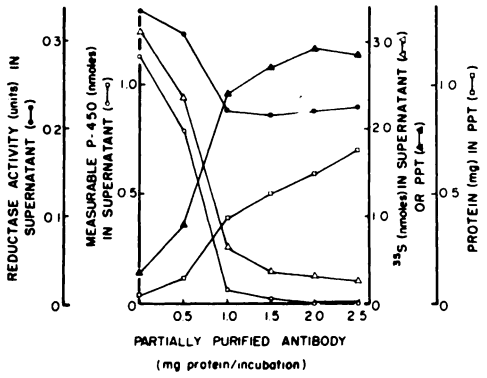

FIG. 1. *Binding of sulfur atom of parathion to both cytochrome P-450 and NADPH-cytochrome c reductase*

The incubation mixture contained 30 nmoles of cytochrome P-450, 7.5 units of NADPH-cytochrome *c* reductase, 120 μg of dilauroyl-L-3-phosphatidylcholine, 100 μg of sodium deoxycholate, 0.015 M $MgCl_2$, 10. mM EDTA, 0.05 M HEPES buffer (pH 7.8), 0.1 mM [^{35}S]parathion, and 0.1 mM NADPH in a final volume of 7.5 ml. After incubation at 37° for 15 min, the mixture was placed in a water-ice bath. The reaction mixture was then dialyzed at 4° for 24 hr against two changes of 50 mM potassium phosphate, pH 7.25 (1 liter), containing 0.85% sodium chloride. A portion of the dialyzed sample was divided equally among six tubes. Each tube contained 1.25 nmoles of cytochrome P-450 as estimated by the method of Omura and Sato (15). Various amounts of partially purified antibody to cytochrome P-450 were added, and the tubes were incubated at 4° for 16 hr. The precipitate was isolated by centrifugation at 2000 × *g*, washed once with 50 mM potassium phosphate, pH 7.25, containing 0.85% sodium chloride, and dissolved in 1 N NaOH. The radioactivity in the supernatant fraction and in the NaOH solution of the precipitate (PPT) was measured by scintillation counting. The NADPH-cytochrome *c* reductase activity in the supernatant was determined using a 50-μl aliquot of each supernatant fraction.

450 antibody precipitate, and in the supernatant after removal of the precipitate, was determined. In addition, the amounts of cytochrome P-450 and NADPH-cytochrome *c* reductase remaining in the supernatant were determined.

As the amount of partially purified antibody was increased, the amounts of radioactivity and cytochrome P-450 in the solution decreased. After 2.0 mg or more of partially purified antibody had been added, cytochrome P-450 was no longer detectable in the supernatant. At this point the radioactivity in the supernatant had decreased by about 90%. This was matched by the appearance of a comparable amount of radioactivity in the antibody-P-450 precipitate. The activity of the reductase in the supernatant decreased by about 30% when 1 mg of partially purified antibody was added. A higher concentration of this antibody preparation had no further effect on the reductase activity in the supernatant. These results suggest that the majority of the nondialyzable sulfur is bound to cytochrome P-450. About 8% of the radiolabeled sulfur remained in the supernatant. This may be bound to the reductase. On the other hand, it may represent sulfur originally bound to cytochrome P-450, which was released in some form into the supernatant during the 16 hr allowed for precipitation of the antibody-P-450 complex.

An experiment similar to that described in Fig. 1 was carried out using whole microsomes isolated from the livers of phenobarbital-treated rats. In these experiments the microsomes were labeled with ^{35}S by incubation with [^{35}S]parathion in the presence of NADPH. The microsomes were then isolated from the incubation mixture by centrifugation, solubilized, and dialyzed to remove unmetabolized parathion and its noncovalently bound metabolites. Then various amounts of partially purified antibody were added to aliquots of the solubilized microsomes, and the amount of radioactivity remaining in the precipitate and in the supernatant, after separation from the antibody-P-450 precipitate, was determined (Fig. 2). The amount of radioactivity in the supernatant decreased with increasing amounts of partially purified antibody. When approximately 4–5 mg of the antibody preparation had been added, the amount of radioactivity remaining in the supernatant was decreased by about 70%. These data suggest that the majority of the sulfur bound to the macromolecules of the microsomes is bound to cytochrome P-450. However, it is quite possible that proteins other than cytochrome P-450 are precipitated, along with P-450, on addition

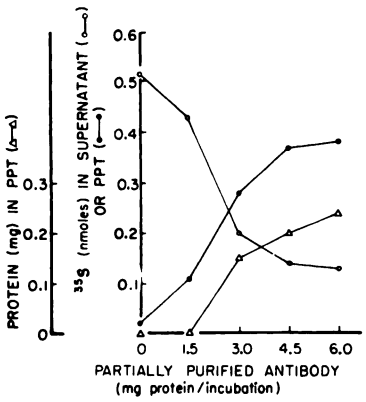

FIG. 2. *Immunoprecipitation analysis of binding of sulfur atom of parathion to cytochrome P-450 in rat liver microsomes*

Microsomes (72 mg of protein) isolated from the livers of phenobarbital-treated rats were incubated for 30 min at 37° with 0.1 mM [^{35}S]parathion, 0.1 mM EDTA, 0.1 mM NADPH, and 0.05 M HEPES buffer, pH 7.8, in a final volume of 36 ml. The incubation mixture was cooled to approximately 4° using a water-ice bath and centrifuged at 105,000 × *g* for 1 hr. The microsomes were dissolved in 10 ml of 100 mM potassium phosphate, pH 7.25, containing 0.6% sodium cholate and 20% glycerol. This solution was centrifuged at 105,000 × *g* for 1 hr. The supernatant was dialyzed at 4° for 3 days against two changes of 50 mM potassium phosphate, pH 7.25 (3.5 liters), containing 0.85% sodium chloride. The dialyzed sample was centrifuged at 5000 × *g* for 20 min, and a portion of it was divided among five tubes, each of which contained 0.164 mg of protein. Various amounts of partially purified antibody to rat liver cytochrome P-450 were then added, and the tubes were incubated as described in Fig. 1. The amount of radioactivity remaining in the supernatant and the precipitate (PPT) was determined as described in Fig. 1.

of the antibody, and these proteins may also be labeled. It has not yet been determined whether the radioactivity remaining in the supernatant is bound to proteins or other macromolecules or is present as some low molecular weight, sulfur-containing compound like HS^- or H_2S. It is possible that the sulfur remaining in the supernatant is bound to a species of cytochrome P-450 not reactive with the partially purified antibody used in these experiments.

Previous work has shown that approximately 50% of the sulfur bound to rat liver microsomes incubated with $C^{35}S_2$ is in the form of a hydrodisulfide (R–S–S–H) (9). This form of bound sulfur was released on treatment of the microsomes with CN^-. The chemical form of the sulfur released was thiocyanate (SCN^-). We therefore examined the ability of CN^- to release the sulfur bound to cytochrome P-450 of the reconstituted system as SCN^- (Figs. 3 and 4). In these experiments a rat liver reconstituted system was incubated with [^{35}S]parathion, dialyzed, and applied to a Sephadex G-25 column to remove the last traces of unmetabolized parathion and its noncovalently bound metabolites. The protein fraction from the Sephadex column was reduced in volume and divided into two portions. One portion was incubated without, and one with, 10 mM CN^- at room temperature for 3 hr. Figure 3 shows the elution profile of protein, radioactivity, and unlabeled SCN^- from a Sepahdex G-25 column of the sample not incubated with CN^-. The unlabeled SCN^- was added just before application to the column. The only peak of radioactivity was that associated with the protein peak. Figure 4 shows the elution profile of the sample incubated with CN^-. There was a decrease in the radioactivity associated with the protein, and a peak appeared which exactly coincided with the colorimetric peak of exogenously added SCN^-. Parathion was eluted from this Sephadex column in fractions 78–105, and the other sulfur-containing metabolite of parathion, diethylphosphorothioic acid, in fractions 35–50. The similarity of these data and those obtained with microsomes labeled with ^{35}S from $C^{35}S_2$, in which the form of the radioactivity released by CN^- was unequivocally identified as SCN^- (9), indicates that a portion of the sulfur bound to proteins of the reconstituted system, and more likely to cytochrome P-450, is present as a hydrodisulfide.

The data in Fig. 1 indicate that the majority of nondialyzable sulfur bound on incubation of parathion with the reconstituted system is bound to cytochrome P-450. Whether the sulfur is bound exclusively to cytochrome P-450 was also examined by means of SDS-polyacrylamide gel

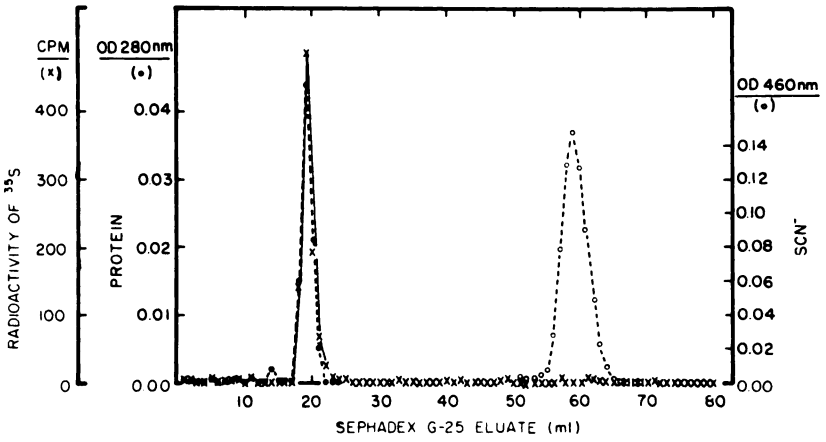

FIG. 3. *Elution profile of absorbance at 280 nm, radioactivity, and SCN^- from Sephadex G-25 to which a ^{35}S-labeled, reconstituted mixed-function oxidase system had been applied*

The 5-ml incubation mixture contained 20 nmoles of cytochrome P-450, 5 units of NADPH-cytochrome c reductase, 500 μg of dilauroyl-L-3-phosphatidylcholine, 500 μg of sodium deoxycholate, and 0.1 mM [^{35}S]parathion. The remainder of the incubation mixture was the same as described in Table 1. The incubation time was 15 min at 37° after a 5-min preliminary incubation. The incubation mixture was next dialyzed at 4° for 24 hr against two changes of 20 mM potassium phosphate, pH 7.8 (1 liter). The reaction mixture was then concentrated to 2 ml using a Minicon Macrosolute concentrator (A-25, Amicon). This concentrated mixture was applied to a Sephadex G-25 column, which was eluted with 20 mM potassium phosphate, pH 7.8. Those fractions containing protein were pooled and concentrated using the Macrosolute concentrator. A portion of the sample (85 μg of protein) was incubated at room temperature for 3 hr, NH_4SCN (1 μmole) was added, and the mixture was applied to the Sephadex G-25 column (1.5 × 28 cm). Fractions of 1 ml were collected. The radioactivity (×——×) represents counts per minute per 0.2 ml. The absorbance at 280 nm (●- - -●) was measured on each 1-ml fraction. Thiocyanate (○- - -○) was determined colorimetrically (17), using 0.5 ml of the appropriate fractions.

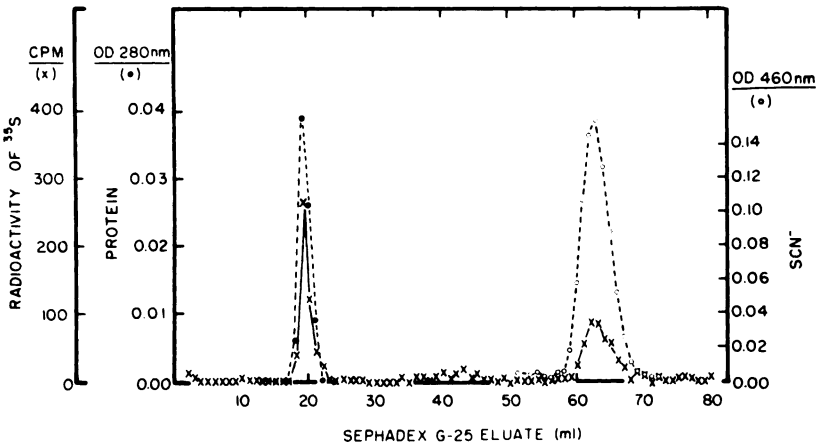

FIG. 4. *Elution profile of protein, radioactivity, and SCN^- from Sephadex G-25 to which a ^{35}S-labeled, reconstituted enzyme system incubated with 10 mM CN^- had been applied*

All incubation conditions and analytical procedures were the same as described in Fig. 3, except that the labeled protein was incubated with 10 mM potassium cyanide for 3 hr at room temperature prior to application to the Sephadex G-25 column. For key to symbols, see Fig. 3.

electrophoresis. In this experiment the reconstituted system was incubated with [^{35}S]parathion, and the reaction mixture was dialyzed and applied to a Sephadex G-25 column to remove the last traces of unreacted parathion and its noncovalently bound metabolites. The protein fraction from the Sephadex column was reduced in volume and divided into two parts. One aliquot was incubated with 100 mM CN^-. Both fractions were then subjected to SDS–polyacrylamide gel electrophoresis in the absence of either dithiothreitol or mercaptoethanol, since these reagents, like CN^-, displace radiosulfur from the labeled proteins. The results are shown in Figs. 5 and 6. Figure 5 shows the results of staining the gels with Coomassie blue. There was considerable protein material at the origin of the gel not treated with CN^- (gel B). The major staining band other than that at the origin was cytochrome P-450. No accumulation of protien at the origin was seen on electrophoresis of the proteins of a reconstituted system incubated with parathion in the absence of NADPH. Electrophoresis of the sample of the sulfur-labeled, reconstituted system which had been incubated with CN^- prior to application to the gel (gel A) showed no protein at the origin. However, either a protein with a molecular weight lower than that of the reductase, impurities in the reductase preparation, or cytochrome P-450 appeared after treatment with CN^-. The origin of this protein is not known at this time.

Figure 6 shows the results of an assay of the radioactivity present in a gel analogous to Fig. 5, gel B. The majority of the radioactivity was associated with the high molecular weight protein at the origin. However, of those proteins which migrated from the origin, only cytochrome P-450 contained any substantial amount of radioactivity. Although not shown, a similar analysis of a gel analogous to Fig. 5, gel A, showed no radioactivity at the origin. Again, the only significant radioactivity detectable on this gel corresponded to the point of migration of cytochrome P-450.

Experiments similar to those described in Fig. 5 were carried out using intact microsomes from phenobarbital-treated rats. In these experiments (Fig. 7) the microsomes were labeled with sulfur by incubation with parathion in the presence of NADPH. The microsomes were isolated by

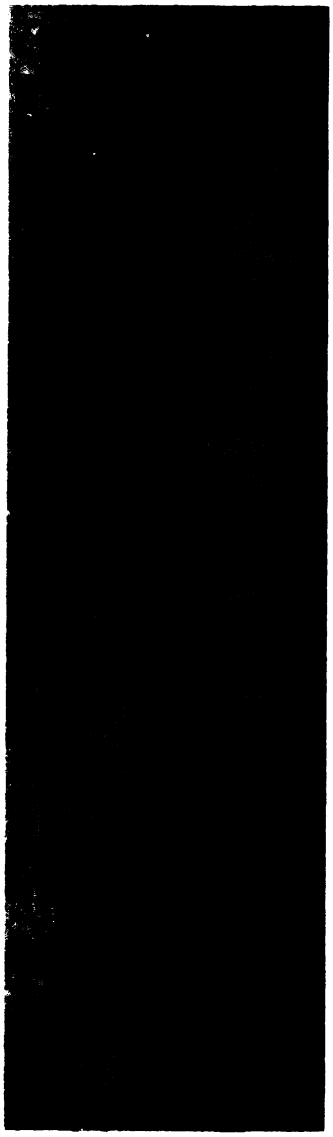

FIG. 5. *SDS-polyacrylamide gel electrophoresis of a ^{35}S-labeled, reconstituted mixed-function oxidase system before (B) and after (A) incubation with CN^-*

The sample for gel B was prepared as described in Fig. 3, and that for gel A was prepared as described in Fig. 4, except that 100 mM rather than 10 mM potassium cyanide was used. Migration is from top to bottom. The conditions for the SDS-gel electrophoresis are described in MATERIALS AND METHODS.

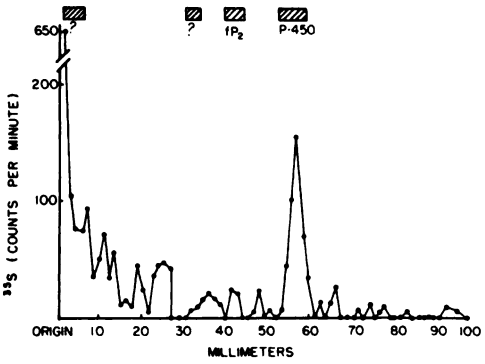

FIG. 6. *Radioactivity in slices of an SDS-polyacrylamide gel resulting from electrophoresis of a ^{35}S-labeled, reconstituted enzyme system*

The sample of protein (approximately 25 μg) which was applied to the gel was prepared as described in Fig. 3. See MATERIALS AND METHODS for details of the SDS-polyacrylamide gel electrophoresis. A schematic illustration of the protein staining of a duplicate gel is shown at the top of the figure (fP_2, flavoprotein). The direction of migration was from left to right.

centrifugation, solubilized in 1% SDS, and subjected to SDS-polyacrylamide gel electrophoresis. The darkly staining band approximately halfway down the gels corresponds to the area of migration of cytochrome(s) P-450. In the gel of the microsomes incubated with [^{35}S]parathion in the presence of NADPH (gel A), a considerable amount of protein remained at the origin. In contrast, the gel of the microsomes incubated with parathion in the absence of NADPH (gel B) showed very little protein at the origin. In other experiments treatment of microsomes, which had been incubated with parathion and NADPH, with CN^- (100 mM), mercaptoethanol (10 mM), or dithiothreitol (10 mM) prior to solubilization and SDS-gel electrophoresis almost completely prevented the accumulation of the high molecular weight protein at the origin. These data are similar to the results described in Fig. 5. Finally, accumu-

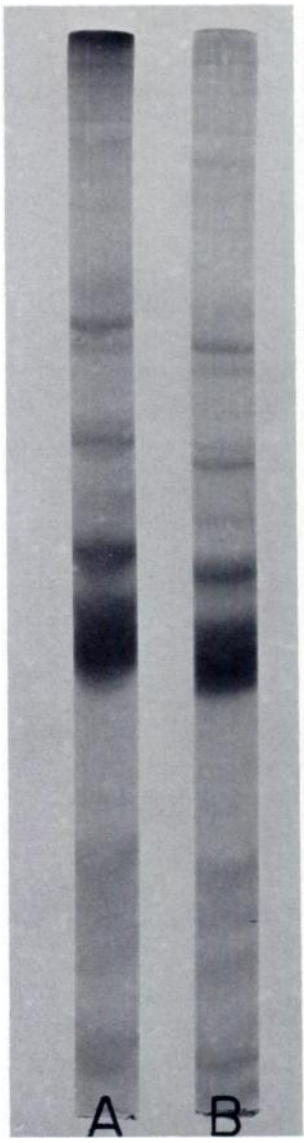

FIG. 7. *SDS-polyacrylamide gel electrophoresis of solubilized rat liver microsomes incubated with [^{35}S]parathion in the presence (A) and absence (B) of NADPH prior to solubilization*

Microsomes (40 mg of protein) isolated from the livers of phenobarbital-treated rats were incubated with [^{35}S]parathion (0.1 mM) in the presence of 0.05 M HEPES buffer (pH 7.8), 0.1 mM EDTA, and 0.1 mM NADPH for 15 min at 37°. The reaction was started by addition of NADPH after preliminary incubation at 37° for 5 min. After incubation the mixture was centrifuged at 105,000 × *g* for 1 hr, and the microsomes were solubilized in 5 ml of 20 mM Tris-acetate, pH 6.0, containing 1% SDS. The solubilized microsomes (40 μg of protein) were then subjected to SDS-polyacrylamide gel electrophoresis as described in MATERIALS AND METHODS. NADPH was omitted from the incubation mixture in the control experiment (gel B). Migration was from top to bottom.

lation of protein at the origin was not seen when microsomes incubated with paraoxon, aniline, or benzphetamine, in the presence of NADPH, were subjected to SDS–polyacrylamide gel electrophoresis as described in Fig. 7. On the other hand, incubation of microsomes with diethyl dithiocarbamate, a compound which dissociates to CS_2 in solution, in the presence of NADPH, led to accumulation of protein at the origin of the gel.

DISCUSSION

Incubation of [^{35}S]parathion with a reconstituted mixed-function oxidase system from rat liver leads to the covalent binding of radioactivity predominantly, if not exclusively, to cytochrome P-450. The data obtained using antibody to rat liver cytochrome P-450 (Fig. 1) and from SDS–polyacrylamide gel electrophoresis (Fig. 6) suggest that there is little, if any, binding of sulfur to the reductase. This covalent binding of radioactivity to cytochrome P-450 is seen only in the presence of a metabolically active, reconstituted system. Companion studies have shown that, compared with [^{35}S]parathion, only 2% as much radioactivity is bound when the complete reconstituted enzyme system is incubated with [*ethyl*-^{14}C]parathion. Considerably less binding of ^{14}C from [*ethyl*-^{14}C]parathion than ^{35}S from [^{35}S]parathion to the macromolecules of rat liver microsomes incubated with [^{14}C]- or [^{35}S]parathion in the presence of NADPH has also been reported (7). These data indicate that the majority of the ^{35}S bound to cytochrome P-450 in the experiments described here is free of the carbon- and phosphorus-containing (7) portions of the parathion molecule. Therefore the sulfur which is bound must be largely that released in the mixed-function oxidase-catalyzed metabolism of parathion to paraoxon. A small portion of the bound sulfur (less than 10%) may be the sulfur atom released in the mixed-function oxidase-catalyzed metabolism of parathion to diethylphosphoric acid (11, 18).

The results of the experiment described in Fig. 2 indicate that the sulfur released in the metabolism of [^{35}S]parathion by intact hepatic microsomes from phenobarbital-treated rats is also bound predominantly to protein(s) which is precipitated by an antibody to cytochrome P-450. However, in these experiments, approximately 30% of the radioactivity remained in the supernatant after addition of saturating amounts of the antibody. It is quite possible that the sulfur which is not precipitated with the P-450 antibody preparation is bound to microsomal macromolecules other than the phenobarbital-induced cytochrome P-450. This may include one or more of the several species of cytochrome P-450 present in microsomes (19–21). These species of cytochrome P-450 may be metabolically active against parathion but not reactive with the antibody preparation used in these experiments (22). Microsomes from rats treated wtih 3-methylcholanthrene are approximately as active in the release and covalent binding of the sulfur atom of [^{35}S]parathion as microsomes from rats treated with phenobarbital (7).

It is also possible that the radiosulfur remaining in the supernatant is present as low molecular weight compounds such as H_2S or HS^-. The data indicating that a portion of the covalently bound sulfur is present as a hydrodisulfide (Figs. 3 and 4) suggest that a portion of the bound sulfur might be released as H_2S or HS^- during prolonged incubation of the solubilized microsomes with the cytochrome P-450 antibody preparation.

The data from the experiments described in Figs. 3 and 4 indicate that approximately 50% of the sulfur atom of parathion bound to cytochrome P-450 of the reconstituted system is in the form of a hydrodisulfide. Similar results were obtained in experiments designed to determine the nature of the bond between the sulfur atom of CS_2 and rat liver microsomes (9). These data also confirm that the form of the sulfur released in the metabolism of parathion to paraoxon is atomic sulfur. The hydrodisulfide linkage is inferred from the ability of the CN^- ion to release the bound sulfur as SCN^-. The enzymes xanthine oxidase (23) and aldehyde oxidase (24) have been reported to

contain a hydrodisulfide linkage in their active sites. This observation was based on the ability of CN^- to release SCN^- from these enzymes. Reaction of synthetic benzyl hydrodisulfide with CN^- also leads to the formation of SCN^- and α-toluenethiol (25). Reaction of parathion or its sulfur-containing metabolite diethylphosphorothioic acid with CN^- does not yield SCN^-. In addition, it is not possible to release SCN^- on reaction of mercaptides like cysteine, glutathione, or dithiothreitol with CN^-. It is only possible to release SCN^- by reaction of CN^- with compounds containing the appropriate polysulfide grouping. Although there may have been binding of more than 1 atom of sulfur to some molecules of P-450 and no binding to others, in the experiments described here (Table 1) approximately 1 nmole of sulfur was bound per nmole of cytochrome P-450. At this binding ratio, the only possible way of forming a hydrodisulfide is by reaction of the side chain of the cysteine contained in cytochrome P-450 ($R-CH_2-S-H$) with atomic sulfur (S) to form the hydrodisulfide ($R-CH_2-S-S-H$). Cytochrome P-450 from rabbit liver contains 6 half-cystines (26). The formation of polysulfides is a common reaction of atomic sulfur. Elemental sulfur exists as S_8, an 8-membered ring of covalently linked sulfur atoms.

Only about 50% of the sulfur bound to cytochrome P-450 can be released as SCN^- (Fig. 4). A similar result was obtained with microsomes labeled with sulfur by incubation with CS_2 in the presence of NADPH (9). Treatment of the microsomes with additional amounts of CN^- or repeated exposure of the microsomes to CN^- did not release the remainder of the bound sulfur (9). It may be that certain hydrodisulfide groups are not accessible to the cyanide ion. However, it is possible that the sulfur is bound by more than one type of linkage. It has been postulated that the sulfur atom of parathion is released in its singlet form (all electrons paired) on reaction of parathion with mixed-function oxidases (27, 28). The singlet sulfur atom participates in carbon-hydrogen insertion reactions in a manner analogous to carbenes and nitrenes (29, 30). It is possible that a portion of the sulfur bound to cytochrome P-450 is the result of carbon-hydrogen insertion reactions. If this is the case, the electronic form of the sulfur released from parathion would most likely be a singlet, since the triplet state of atomic sulfur does not readily participate in carbon-hydrogen insertion reactions.

The formation of a high molecular weight aggregate of the proteins of the reconstituted system and of proteins in microsomes on incubation with parathion is of considerable interest. The ability to dissociate this high molecular weight material using nucleophiles such as CN^-, mercaptoethanol, or dithiothreitol implies that the complex may be formed by linking macromolecules by way of polysulfide chains involving one or more of the hydrodisulfides formed on binding of the sulfur atoms. This is supported by the observation that, in contrast to the results seen with parathion and diethyl dithiocarbamate, no high molecular weight material is formed when microsomes are incubated with benzphetamine, paraoxon, or aniline in the presence of NADPH, or with parathion in the absence of NADPH.

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