Biochemical Pharmacology, Vol. 25, pp. 37–45. Pergamon Press, 1976. Printed in Great Britain.

EXAMINATION OF THE METABOLISM *IN VITRO* OF PARATHION (DIETHYL *p*-NITROPHENYL PHOSPHOROTHIONATE) BY RAT LUNG AND BRAIN*

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(*Received* 9 *December* 1974; *accepted* 4 *April* 1975)

Abstract— These studies have indicated there is present in rat lung microsomes a mixed-function oxidase enzyme system capable of metabolizing parathion to paraoxon and to diethyl phosphorothioic acid. In addition, analogous to previous results using rat liver microsomes, the sulfur atom released in the metabolism of parathion to paraoxon was found to covalently bind to lung microsomes. In contrast to liver, the metabolism of parathion by rat lung microsomes is not inducible by pretreatment of the animals with phenobarbital or 3-methylcholanthrene. The metabolism of parathion by lung microsomes is stimulated by NADPH and oxygen and is inhibited by carbon monoxide, anaerobic conditions, SKF-525A and piperonyl butoxide. There is also present in a particulate fraction of rat brain equivalent to microsomes an enzyme or enzyme system capable of metabolizing parathion to paraoxon and to diethyl phosphorothioic acid. The metabolism of parathion to paraoxon by rat brain microsomes is also accompanied by the release and covalent binding of the sulfur atom of parathion. The activity in rat brain microsomes is stimulated by oxygen and NADPH and inhibited by carbon monoxide, anaerobic conditions, SKF-525A and piperonyl butoxide. These data suggest that cytochrome P-450-containing mixed-function oxidase enzyme systems are responsible for the NADPH-stimulated catalytic activity toward parathion found in rat lung and rat brain microsomes.

The organophosphate insecticide parathion (diethyl *p*-nitrophenyl phosphorothionate) is metabolized by mammalian hepatic mixed-function oxidase enzyme systems to its toxic metabolite paraoxon (diethyl *p*-nitrophenyl phosphate) [1–3] and to its essentially nontoxic metabolites diethyl phosphorothioic acid plus *p*-nitrophenol [4,5].

The principal cause of death in mammals exposed to parathion is respiratory failure. It has been generally assumed that the sequence of events leading to respiratory failure is the hepatic mixed-function oxidase catalyzed metabolism of parathion to paraoxon, a potent inhibitor of acetylcholinesterase, followed by transfer of the paraoxon via the circulatory system to the lung and brain, where inhibition of this enzyme results in respiratory failure. However, it has recently been suggested that the metabolism of parathion and other cholinergic phosphorothionate triesters to their toxic oxygen analogues in extrahepatic tissue may be of greater importance in regards to the toxicity of this compound [6,7].

There have been relatively few studies of the extrahepatic metabolism of parathion in mammals. Kubišťova [8], using an anticholinesterase assay for paraoxon, found evidence for the metabolism *in vitro* of parathion to paraoxon in tissue slices from the small intestine, lung, kidney and suprarenal gland of the adult female rat. However, no activity was detectable in slices of heart muscle, diaphragm, spleen, pancreas, brain or ovaries. Neal [4] and Nakatsugawa *et al.* [9], using 9000 *g* supernatants and whole homogenates, respectively, of various tissues of the rat, were able to detect significant metabolic activity toward parathion in the lung and kidney but found only questionable activity in the brain, intestine, spleen, pancreas and heart. Alary and Brodeur [10], using homogenates of various tissues of the rats, detected parathion-metabolizing activity in the intestine, lung and kidney but not in the brain, serum or skeletal muscle.

Of the various extrahepatic tissues, the metabolism of parathion to paraoxon in the brain and lungs is most likely to be of importance in regard to its toxicity [6]. The lung appears to be a particularly important tissue in this regard since the cause of death in acute poisoning is respiratory failure resulting from the paralysis of the muscles of respiration, bronchioconstriction, accumulation of fluid in the lungs as well as central nervous system effects [11].

Although detailed studies of the metabolism of parathion by rabbit lung microsomes have been carried out [6], no comparable studies using rat lung microsomes have yet been conducted. The purpose of this study was to examine in detail the metabolism of parathion by rat lung microsomes. In addition, a particulate fraction from rat brain equivalent to microsomes was examined for its ability to metabolize parathion.

METHODS

The ethyl-[^{14}C]- and [^{35}S]parathion (6–10 μCi/μmole) used in these studies were products of the

* This work was supported by National Institutes of Environmental Health Sciences Grants ES 00075 and ES 00267. The training support of NIEHS Grant ES 00112 is also gratefully acknowledged.

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Amersham-Searle Corp. NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were products of Boehringer-Mannheim. SKF-525A was a gift of Smith, Kline & French. Piperonyl butoxide was obtained from the K & K Laboratories.

Adult male Sprague–Dawley rats weighing between 150 and 250 g were used in these studies. Pooled lungs from eight rats were homogenized in 3 vol. ice-cold 0·25 M sucrose, first in a Waring blender (10 sec at top speed) and then in an all glass Potter–Elvehjem homogenizer using twelve passes. The pooled brains of eight rats were homogenized in 0·25 M sucrose using eight passes of a Teflon-glass Potter–Elvehjem homogenizer. These homogenates were centrifuged at 9000 *g* for 10 min. The supernatants were removed using a large syringe and recentrifuged at 9000 *g* for 10 min. The resulting supernatant was centrifuged at 250,000 *g* for 30 min. The precipitates from these centrifugations were resuspended in 0·25 M sucrose and centrifuged again at 250,000 *g* for 30 min. The precipitates were resuspended in 0·05 M Hepes buffer (pH 7·8) and used immediately. All operations were carried out at 0–4°. No attempt was made to determine the homogeneity of these preparations as regards contamination with particulate matter other than endoplasmic reticulum. However, for the sake of simplicity, the precipitates from the centrifugation at 250,000 *g* will be referred to throughout the remainder of this manuscript as microsomes.

Microsomes isolated from the lungs and brains were incubated in 0·05 M Hepes buffer (pH 7·8) with labeled parathion in the absence or presence of an NADPH-generating system (5 μmoles NADP, 15 μmoles glucose 6-phosphate and 1 unit glucose 6-phosphate dehydrogenase). The incubations were carried out in serum stoppered 50-ml Erlenmeyer flasks at 37° using a reciprocating shaker bath. The total volume of the incubations was 2·0 ml containing 1·5 to 2·5 mg brain or lung microsomal protein/ml. Parathion concentrations of 5×10^{-5} and 15×10^{-5} M were used in the incubations containing lung and brain microsomes respectively. The incubation time was 2 min after a 5-min temperature equilibration period using lung microsomes and 5 min after a 5-min temperature equilibration period using brain microsomes. In both cases the reactions were started by injecting parathion dissolved in 50 μl methanol through the stopper and terminated by injecting 100 μl concentrated HCl. The procedures used for measuring paraoxon and diethyl phosphorothioic acid formation as well as those used to quantitate the covalent binding of sulfur to the microsomes have been described previously [4,12].

In the incubations examining the effect of atmospheres of carbon monoxide and nitrogen on the rates of metabolism of parathion, serum stoppered 50-ml Erlenmeyer flasks containing microsomes and the NADPH-generating system, if applicable, were held on ice, and the appropriate mixture of carbon monoxide and air or nitrogen and air was bubbled through the solutions for 5 min. After a 5-min period of temperature equilibration, the reactions were started by injecting labeled parathion dissolved in methanol through the stopper. Oxygen-free nitrogen and carbon monoxide were obtained by passing these gases through deoxygenating towers, each of which contained 200 ml of 0·09% sodium anthroquinone-2-sulfonate and 5% sodium dithionite in 0·1 N NaOH.

Rats pretreated with phenobarbital received intraperitoneal injections of 50 mg/kg of sodium phenobarbital in distilled water daily for 5 days followed by sacrifice 24 hr after the last injection. Animals pretreated with 3-methylcholanthrene received a single intraperitoneal injection of 20 mg/kg of 3-methylcholanthrene in corn oil followed by sacrifice 72 hr after the injection.

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 this purpose [13]. The standard normal deviate test was used to test for significant differences between the apparent K_m and V_{max} values.

RESULTS

Various particulate fractions of rat lung were examined for their ability to metabolize parathion to paraoxon and diethyl phosphorothioic acid. In these studies the rat lung was homogenized in 3 vol. of 0·05 M Hepes buffer, and the supernatant remaining after a 20-min centrifugation of the homogenate at 600 *g* (600 *g* supernatant), the precipitate from a 10-min centrifugation at 8000 *g* (mitochondria), the precipitate from a centrifugation of the 8000 *g* supernatant for 30 min at 250,000 *g* (microsomes) and the supernatant from the final centrifugation (soluble) were incubated with [^{14}C]parathion as described in Methods. The results are shown in Table 1. As can be seen, the rat lung is capable of catalyzing the metabolism of parathion to paraoxon and diethyl phosphorothioic acid. In addition, the data in Table 1 indicate that the enzyme activity is located primarily in the microsomal fraction. The small amount of activity in the mitochondrial and soluble fractions is thought to be the result of microsomal contamination of these fractions. However, no attempt was made

Table 1. Examination of the rate of formation of paraoxon and diethyl phosphorothioic acid using various particulate fractions of the lungs of adult male rats*

Cell fraction	Metabolite† (pmoles formed/min/0·5 g wet wt lung) Paraoxon	Diethyl phosphorothioic acid
600 *g* Supernatant	911 ± 119	566 ± 51
Mitochondria	199 ± 9	88 ± 16
Microsomes	711 ± 101	243 ± 0
Soluble	132 ± 19	55 ± 2

* A 5×10^{-5} M concentration of [^{14}C]parathion was incubated with the various particulate fractions equivalent to 0·5 g wet wt of the pooled lungs of eight rats for 2 min as described in Methods. All incubations contained the NADPH-generating system described in Methods. The rates of formation of paraoxon and diethyl phosphorothioic acid were estimated as described previously [12].

† Means ± standard deviations of the means of duplicate determinations using microsomes isolated from a pooled sample of eight rat lungs.

Table 2. Effect of the presence or absence of NADPH on ^{35}S binding, paraoxon and diethyl phosphorothioic acid formation*

Reaction measured (pmoles/min/mg protein)	NADPH† Plus	Minus
^{35}S binding	171 ± 10	14 ± 0
Paraoxon formation	158 ± 4	5 ± 0
Diethyl phosphorothioic acid formation	36 ± 2	1 ± 0

* The 5×10^{-5} M concentrations of [^{35}S]- (determination of ^{35}S binding) or [^{14}C]parathion (determination of paraoxon and diethyl phosphorothioic acid formation) were incubated with microsomes isolated from the pooled lungs of eight adult male rats in the presence and absence of an NADPH-generating system, and the rates of ^{35}S binding, paraoxon and diethyl phosphorothioic acid formation determined as described in Methods. The procedures used to estimate metabolite formation or sulfur binding have been described previously [12].

† Means ± standard deviations of the means of duplicate determinations using microsomes isolated from a pooled sample of eight rat lungs.

to verify this by measurement of the activity of microsomal marked enzymes in these fractions. All subsequent studies with rat lung were carried out using the microsomal fraction.

When [^{35}S]parathion is incubated with microsomes isolated from rat liver in the presence of NADPH, an amount of sulfur, roughly equivalent to the amount of paraoxon formed in these incubations, becomes covalently bound to the microsomes [12]. It has been shown that the bound sulfur is that which is released in the mixed-function oxidase catalyzed metabolism of parathion to paraoxon. These studies have also shown that the rate of the mixed-function oxidase catalyzed metabolism of parathion to paraoxon may be estimated by measuring either the amount of paraoxon in the incubation mixture at the end of the incubation or the amount of sulfur covalently bound to the microsomes. In light of these results, it was of interest to incubate [^{35}S]parathion with lung microsomes, in the presence and absence of NADPH, and to compare the rate of sulfur binding to these microsomes with the rate of formation of paraoxon in equivalent incubations containing [^{14}C]parathion. In addition, the rate of formation of diethyl phosphorothioic acid in the incubations containing [^{14}C]parathion was also measured. The results of these experiments are shown in Table 2. In all cases, the rates of the three reactions in the absence of NADPH were less than 10 per cent of those seen in the presence of NADPH. Consequently, the rates of these reactions in the absence of NADPH were disregarded in subsequent experiments. Analogous to the results obtained using rat liver microsomes [12], the rate of sulfur binding was very similar to that of paraoxon formation using equal aliquots of the same preparation of lung microsomes.

[^{14}C]parathion was incubated with lung microsomes in the presence of NADPH, and the amount of [^{14}C] covalently bound was compared with the amount of [^{35}S] bound in an equivalent incubation containing [^{35}S]parathion. The results showed that the amount of [^{14}C] bound was only 10 per cent that of [^{35}S] (data not shown). Thus, the majority, if not all, of the sulfur bound to the lung microsomes is free of the remainder of the parathion molecule.

Using rat liver microsomes [14] or rabbit lung microsomes [6], the rate of formation of diethyl phosphorothioic acid is approximately 50 per cent that of paraoxon formation. As shown in Table 2, rat lung microsomes appear to metabolize parathion to diethyl phosphorothioic acid at a rate which is only about 25 per cent that of paraoxon formation.

The pH optimum for the metabolism of parathion to paraoxon using rat lung microsomes was examined. The results showed a broad pH optimum with the maximal rate occurring between pH values of 7 and 8·5.

The effect of heating the rat lung microsomes on their ability to metabolize parathion to paraoxon was also examined. It was found that heating of the microsomes to 100° for 5 min completely abolished the NADPH-stimulated metabolism of parathion to paraoxon.

The apparent K_m and V_{max} values for parathion metabolism by rat lung microsomes as measured by the rate of sulfur binding, paraoxon and diethyl phosphorothioic acid formation are given in Table 3. There is not a statistically significant difference between the apparent K_m or V_{max} values for paraoxon formation and the analogous kinetic constants for sulfur binding. As was the case with liver microsomes [12], the sulfur bound when parathion is incubated with rat lung microsomes is most likely that released in the metabolism of parathion to paraoxon. Also, analogous to the experiment using hepatic microsomes [12], the rate of binding of sulfur to lung microsomes is apparently equal to or greater than the rate-limiting step in the series of reactions leading to the release of sulfur from parathion. The data shown in Tables 2 and 3 indicate that the rate of metabolism of parathion to paraoxon by rat lung can be estimated by measuring either sulfur binding or paraoxon formation. The measurement of sulfur binding has the advantage of being faster and requiring less expenditure of effort than the measurement of the

Table 3. Apparent K_m and V_{max} values for the metabolism of parathion to diethyl phosphorothioic acid and to paraoxon measuring either ^{35}S binding or paraoxon formation directly*

Reaction measured	Apparent K_m ($\times 10^{-6}$ M)	V_{max} (pmoles/min/mg protein)
^{35}S binding	5·3 ± 1·8	184 ± 15
Paraoxon formation	7·9 ± 2·1	206 ± 25
Diethyl phosphorothioic acid formation	9·0 ± 4·2	57 ± 7

* [^{35}S]- or [^{14}C]-labeled parathion in concentrations of $0{\cdot}25 \times 10^{-5}$ M, $0{\cdot}5 \times 10^{-5}$ M, 1×10^{-5} M, 2×10^{-5} M and 4×10^{-5} M was incubated in duplicate for 2 min with microsomes isolated from the pooled lungs of eight adult male Sprague–Dawley rats as described in Methods. The procedures used to estimate the rate of ^{35}S binding, paraoxon and diethyl phosphorothioic acid formation have been described previously [12]. The apparent K_m and V_{max} values were determined as described in Methods.

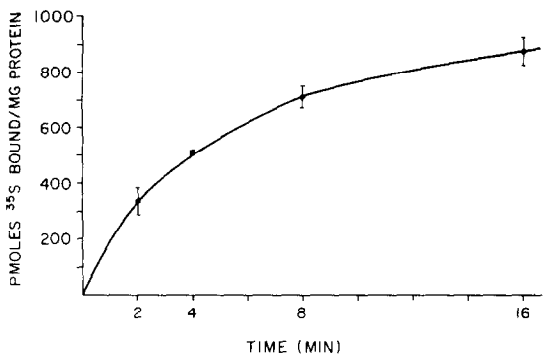

Fig. 1. Linearity with time of parathion metabolism by rat lung microsomes as measured by sulfur binding. Each point represents the mean ± standard deviation of the means of duplicate determinations at each time point. [^{35}S]parathion (5×10^{-5} M) was incubated with lung microsomes and an NADPH-generating system as described in Methods. The procedures used to estimate the sulfur binding have been described previously [12].

amount of paraoxon present at the end of the incubation period.

The linearity of parathion metabolism with time as measured by sulfur binding to lung microsomes is shown in Fig. 1. The reaction appears to be linear for only 2 min or less. Because of the technical difficulties involved in carrying out the simultaneous incubation of a number of samples with reaction times of less than 2 min, the experiments described in this paper were carried out using incubation times of 2 min.

Figure 2 compares the amount of sulfur bound to lung microsomes using increasing concentrations of microsomal protein and a constant amount of [^{35}S]parathion (5×10^{-5} M). As can be seen, the binding of sulfur is linear in the range of 0·5 to 3·0 mg protein/ml. A range of 1·5 to 2·5 mg lung microsomal protein/ml was used throughout these studies.

The effect of pretreatment of rats with phenobarbital or 3-methylcholanthrene on the NADPH-stimulated release and binding of labeled sulfur to rat lung microsomes was examined. The data from these experiments indicated there were no significant differences in the rate of the release and covalent binding of the labeled sulfur to microsomes isolated from the phenobarbital- or 3-methylcholanthrene-pretreated animals compared to untreated. It thus appears that, in contrast with rat liver microsomes [12], the enzyme or enzyme system in rat lung microsomes which catalyzes the metabolism of parathion to paraoxon with the release of a reactive form of sulfur is not subject to induction by phenobarbital or 3-methylcholanthrene. An experiment in which the rates of paraoxon and diethyl phosphorothioic acid formation were measured was also carried out. Again, no significant difference in the rate of metabolism of parathion to these two metabolites by lung microsomes was detected in animals pretreated with phenobarbital or 3-methylcholanthrene compared to untreated.

The effect of atmospheres containing varying concentrations of carbon monoxide, nitrogen and oxygen on the rate of sulfur binding and the rate of formation of paraoxon and diethyl phosphorothioic acid using rat lung microsomes is shown in Table 4. When the

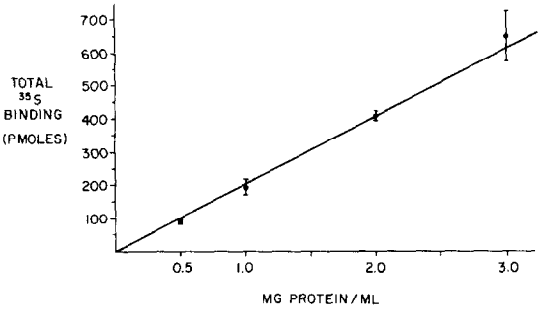

Fig. 2. Examination of sulfur binding using increasing lung microsomal protein concentrations. Each point represents the mean ± standard deviation of the means of duplicate determinations at each protein concentration. The incubation time was 2 min and the [^{35}S]parathion concentration was 5×10^{-5} M. The data are presented as the total pmoles of ^{35}S bound/min at each protein concentration.

Table 4. Effect of atmospheres of carbon monoxide, nitrogen and oxygen compared to air on the binding of ^{35}S and the formation of paraoxon and diethyl phosphorothioic acid*

Atmosphere	^{35}S binding†	Paraoxon formation†	Diethyl phosphorothioic acid formation†
	(pmoles/min/mg protein)		
Air	197 ± 10	169 ± 3	51 ± 1
CO air (90 : 10)	37 ± 6	32 ± 9	12 ± 0
N_2 air (90 : 10)	221 ± 2	180 ± 24	73 ± 1
N_2	26 ± 3	15 ± 4	4 ± 0
O_2	147 ± 7	129 ± 13	31 ± 3

* The atmospheres were created as described in Methods. Incubations were done with 5×10^{-5} M [^{35}S]parathion to estimate the rate of ^{35}S binding and with 5×10^{-5} M [^{14}C]parathion to estimate the rate of paraoxon and diethyl phosphorothioic acid formation. The incubation procedures are described in Methods. The procedures used to estimate sulfur binding, paraoxon and diethyl phosphorothioic acid formation have been described previously [12].

† Means ± standard deviations of the means of duplicate determinations using microsomes isolated from a pooled sample of eight adult male rat lungs.

Table 5. Effect of SKF-525A, piperonyl butoxide and benzopyrene on the metabolism of parathion to paraoxon*

Additions	Paraoxon formation† (pmoles/min/mg protein)	% Inhibition
None	148 ± 21	
SKF-525A (3 mM)	62 ± 0	58
Piperonyl butoxide (1 mM)	87 ± 1	41
3,4-Benzopyrene (1 mM)	147 ± 1	0

* Acetone (0·1 ml) was used as the solvent for the inhibitors. An equivalent amount of acetone was added to the incubations containing no inhibitor. [^{14}C]parathion (5×10^{-5} M) was incubated with lung microsomes in the presence and absence of the inhibitors. In these experiments, both the inhibitors and parathion were present in the incubation mixture along with lung microsomes during the 5-min temperature equilibration period. The reactions were started by adding the NADPH-generating system. Other details of these incubations are described in Methods. The procedures used to estimate paraoxon formation have been described previously [12].

† Means ± standard deviations of the means of duplicate determinations using microsomes isolated from a pooled sample of eight adult male rat lungs.

incubations were carried out in an atmosphere containing carbon monoxide–air (90 : 10), the rate of binding of labeled sulfur as well as the rate of formation of paraoxon and diethyl phosphorothioic acid was strongly inhibited compared to those incubations carried out in air. When the lung microsomes were incubated with labeled parathion in an atmosphere of nitrogen–air (90 : 10) the rate of formation of these three metabolites was consistently increased compared to incubations carried out in air. On the other hand, when the incubations were carried out in oxygen-free nitrogen, the rate of metabolism of parathion was strongly inhibited. When the incubations were carried out in an atmosphere of pure oxygen, the rate of formation of these three metabolites was decreased in comparison with incubations carried out in air. These data suggest that some optimal level of oxygen is required for the maximal rates of metabolism of parathion by lung microsomes and that concentrations above and below this level inhibit these reactions.

The mixed-function oxidase catalyzed metabolism of parathion using rat liver microsomes is inhibited when the incubation is carried out in the presence of certain alternate substrates for this enzyme system [14]. Two of these inhibitory alternate substrates are SKF-525A [14] and piperonyl butoxide (R. A. Neal, unpublished observations). The ability of SKF-525A or piperonyl butoxide to inhibit the metabolism of parathion by lung microsomes was examined. In addition, the ability of 3,4-benzopyrene to inhibit parathion metabolism by lung microsomes was also examined. The results of these studies are shown in Table 5. As can be seen, both SKF-525A and piperonyl butoxide inhibited the metabolism of parathion by lung microsomes. However, 3,4-benzopyrene, a good substrate for the mixed-function oxidase system referred to as arylhydrocarbon hydroxylase, did not exhibit any inhibitory effect on parathion metabolism. These latter data suggest that the enzyme system in rat lung which is responsible for parathion metabolism is distinct from arylhydrocarbon hydroxylase.

It has been shown previously that sulfur becomes covalently bound to brain tissue after administration *in vivo* of [^{35}S]parathion to adult male rats [15]. It was, therefore, of interest to examine if the metabolism of parathion to paraoxon and to diethyl phosphorothioic acid could be demonstrated using a particulate fraction from rat brain equivalent to microsomes. In addition, the ability of brain microsomes to catalyze the release and covalent binding of the sulfur atom of parathion was also examined. The results of these experiments are shown in Table 6. From these data, it appears that there is a measurable conversion of parathion to paraoxon and to diethyl phosphorothioic acid in the absence of NADPH. However, in the presence of NADPH there was a small but significant increase in the formation of these two metabolites. The data from the experiment measuring the rate of labeled sulfur binding are nearly identical to those seen when the formation of paraoxon was measured.

An examination of the effect of pH on the NADPH-stimulated metabolism of parathion to paraoxon by rat brain microsomes revealed a broad pH optimum with the maximum rate occurring between pH values of 7·2 and 8·5.

The linearity with time of the NADPH-stimulated parathion metabolism by brain microsomes, as measured by sulfur binding, is shown in Fig. 3. The reaction appears to be nearly linear for about 5 min. Consequently, a 5-min incubation time was used in those experiments involving rat brain microsomes.

The apparent K_m and V_{max} values for the NADPH-stimulated metabolism of parathion by rat brain microsomes as measured by sulfur binding, paraoxon and diethyl phosphorothioic acid formation are shown in Table 7. The apparent K_m or V_{max} values for the NADPH-stimulated sulfur binding using rat brain microsomes are not significantly different from these same constants for paraoxon formation. This suggests

Table 6. Effect of the presence and absence of NADPH on ^{35}S binding, paraoxon and diethyl phosphorothioic acid formation*

Reaction measured (pmoles/min/mg protein)	NADPH†	
	Plus	Minus
^{35}S binding	72 ± 1	48 ± 4
Paraoxon formation	78 ± 2	48 ± 0
Diethyl phosphorothioic acid formation	6 ± 1	2 ± 1

* The 15×10^{-5} M concentrations of ^{35}S- (sulfur binding) or [^{14}C]parathion (paraoxon and diethyl phosphorothioic acid formation) were incubated with microsomes isolated from the pooled brains of eight adult male rats in the presence and absence of an NADPH-generating system as described in Methods. The procedures used to estimate ^{35}S binding, paraoxon and diethyl phosphorothioic acid formation have been described previously [12].

† Means ± standard deviations of the means of duplicate determinations using microsomes isolated from a pooled sample of eight rat brains.

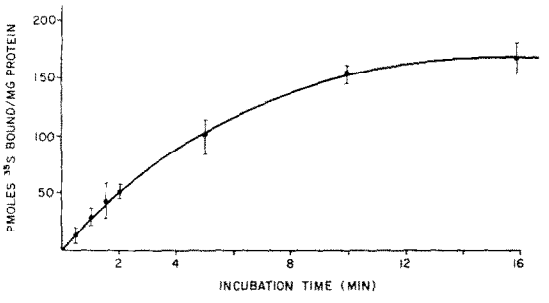

Fig. 3. Linearity with time of parathion metabolism by rat brain microsomes as measured by NADPH-stimulated sulfur binding. Each point represents the mean ± standard deviation of the means of two determinations. [^{35}S]parathion (15×10^{-5} M) was incubated with brain microsomes for varying time periods in the presence and absence of NADPH as described in Methods. The NADPH-stimulated binding of sulfur in each incubation was determined by subtracting the amount bound in the absence from that bound in the presence of an NADPH-generating system.

that as was the case with rat liver [12] and rat lung microsomes, the sulfur bound to brain microsomes is that released in the metabolism of parathion to paraoxon.

The effect of pretreatment of rats with phenobarbital and 3-methylcholanthrene *in vivo* on the rate of NADPH-stimulated binding of sulfur to brain microsomes *in vitro* was also examined. As with rat lung microsomes, there was not a significant difference in the rate of labeled sulfur binding to the microsomes of the brains of the phenobarbital-pretreated and 3-methylcholanthrene-pretreated animals compared to untreated (data not shown).

The effect of incubation of parathion with rat brain microsomes in atmospheres containing varying concentrations of carbon monoxide, nitrogen and oxygen is shown in Table 8. Compared to incubations carried out in air, incubations conducted in atmospheres of 90 : 10 carbon monoxide–air or nitrogen–air had no significant effect on the rate of NADPH-stimulated

Table 7. Apparent K_m and V_{max} values for the NADPH-stimulated metabolism of parathion to paraoxon and to diethyl phosphorothioic acid*

Reaction measured	K_m ($\times 10^{-5}$ M)	V_{max} (pmoles/min/mg protein)
^{35}S binding	3·0 ± 0·3	40 ± 2
Paraoxon formation	2·4 ± 0·3	41 ± 7
Diethyl phosphorothioic acid formation	5·9 ± 0·9	6 ± 1

* [^{35}S]- or [^{14}C]-labeled parathion in concentrations of 1×10^{-5} M, 2×10^{-5} M, 4×10^{-5} M and 8×10^{-5} M was incubated in duplicate for 5 min in the presence and absence of an NADPH-generating system, with microsomes isolated from the pooled brains of eight adult male Sprague–Dawley rats as described in Methods. The amounts of sulfur bound, paraoxon or diethyl phosphorothioic acid formed in the absence of NADPH were subtracted from the amounts found in the presence of NADPH and the differences used to calculate the apparent K_m and V_{max} values.

Table 8. Effect of atmospheres of nitrogen, carbon monoxide and oxygen, compared to air, on paraoxon formation*

Atmosphere	NADPH-stimulated paraoxon formation† (pmoles/min/mg protein)
Air	46 ± 10
N_2–air (90 : 10)	59 ± 10
CO–air (90 : 10)	50 ± 11
N_2–air (99 : 1)	101 ± 20
CO–air (99 : 1)	8 ± 2
N_2	10 ± 8
O_2	24 ± 5

* The various atmospheres were created as described in Methods. The incubations were carried out using 15×10^{-5} M concentrations of [^{14}C]parathion as described in Methods and paraoxon formation was estimated as described previously [12].

† Means ± standard deviations of the means of duplicate determinations using microsomes isolated from a pooled sample of eight adult male rat brains. The values represent the difference in the rate of formation of paraoxon in the presence compared to the absence of an NADPH-generating system.

metabolism of parathion to paraoxon. However, when the incubations were carried out in an atmosphere of 99 : 1 nitrogen–air there was a significant increase in the rate of paraoxon formation in the presence but not the absence of NADPH. In contrast, when the incubations were carried out in an atmosphere of 99 : 1 carbon monoxide–air there was a significant decrease in the rate of metabolism of parathion to paraoxon in the presence of NADPH. This concentration of carbon monoxide did not affect the rate of formation of paraoxon in the absence of NADPH. When the incubations were carried out in an atmosphere of oxygen-free nitrogen, there was a marked inhibition of the rate of metabolism of parathion to paraoxon both in the presence and, to a lesser extent, in the absence of NADPH. When the reaction was carried out in an atmosphere of 100% oxygen, the rate in the presence of NADPH was inhibited. These data indicate that there is a requirement for oxygen for the NADPH-stimulated metabolism of parathion to paraoxon by rat brain microsomes, although the concentration of oxygen required for the optimal rate of this reaction is low.

If, as the previous results suggest, the NADPH-stimulated metabolism of parathion by brain microsomes is catalyzed by a cytochrome P-450-containing enzyme system, it appeared possible that SKF-525A and piperonyl butoxide might be inhibitors of these reactions. Recall that these compounds are inhibitors of parathion metabolism by rat liver [14] and rat lung. Table 9 shows the effect of simultaneous incubation of SKF-525A or piperonyl butoxide and parathion with rat brain microsomes on the rate of formation of paraoxon from parathion. The NADPH-stimulated rate of paraoxon formation by rat brain microsomes was markedly or completely inhibited in those reactions containing both SKF-525A or piperonyl butoxide and [^{14}C]parathion. The rate of formation of paraoxon seen in the absence of NADPH was not affected by the presence of SKF-525A or piperonyl butoxide.

Table 9. Effect of SKF-525A and piperonyl butoxide on the metabolism *in vitro* of parathion to paraoxon by rat brain microsomes*

Addition	NADPH stimulated paraoxon formation† (pmoles/min/mg protein)	% Inhibition
None	44 ± 4	
SKF-525A (3 mM)	2 ± 0	95
Piperonyl butoxide (1 mM)	0 ± 0	100

* Acetone was used as the solvent for the inhibitors. An equivalent amount of acetone was added to the incubations containing no inhibitor. [^{14}C]parathion (15×10^{-5} M) was incubated with brain microsomes in the presence and absence of the inhibitors and in the presence and absence of an NADPH-generating system as described in Methods. Additional details of the procedures used in these experiments are described in Table 5.

† Means ± standard deviations of the means of duplicate determinations using microsomes isolated from a pooled sample of eight rat brains. These values represent the difference in the rate of paraoxon formation in the presence compared to the absence of an NADPH-generating system.

When the brain microsomes were heated to 100° for 5 min prior to incubation with parathion, the NADPH-stimulated metabolism of parathion to paraoxon was completely eliminated. However, the heating of the microsomes did not affect the conversion of parathion to paraoxon seen in the absence of NADPH.

DISCUSSION

The results of these experiments have shown that parathion is metabolized by rat lung and rat brain microsomes in a manner which is qualitatively similar to that previously shown using rat liver microsomes [4,5]. As with liver microsomes, both paraoxon and diethyl phosphorothioic acid are products of the NADPH-stimulated metabolism of parathion by rat lung and rat brain microsomes. There are some major quantitative differences, however. The maximal rates of metabolism of parathion by rat lung and rat brain microsomes are about 20 and 3 per cent respectively, of those seen using rat liver microsomes when the rates are calculated on the basis of microsomal protein concentration. In addition, a lesser percentage of the parathion is metabolized to diethyl phosphorothioic acid by rat lung and rat brain microsomes compared to rat liver microsomes.

The apparent K_m values for parathion for metabolism to paraoxon and to diethyl phosphorothioic acid using rat brain microsomes are quite similar to those determined using rat liver microsomes [12,14]. However, the K_m values for parathion for formation of these two metabolites using rat lung microsomes are about five times less than those determined using rat liver microsomes. The higher affinity of the enzyme system in rat lung microsomes for parathion may be of importance concerning the comparative rate of metabolism of parathion to paraoxon *in vivo*. Using saturating substrate concentrations, the rate of metabolism of parathion to paraoxon by rat liver microsomes is about five times that using rat lung microsomes. However, under conditions *in vivo* one would not expect to achieve saturating parathion concentrations at the active sites of these enzymes. Therefore, the differential in the rate of metabolism of parathion by the endoplasmic reticulum in these two organs *in vivo* would probably be less than that seen at saturating substrate concentrations under conditions *in vitro*. This, of course, assumes that the affinity for parathion determined *in vitro* is similar to that *in vivo*, a question which it is not possible to answer.

The metabolism of parathion by both rat lung and rat brain microsomes is stimulated by NADPH and inhibited by carbon monoxide, by anaerobic conditions and by SKF-525A and piperonyl butoxide, both of which are substrates for the hepatic mixed-function oxidase enzyme system. These data, coupled with the fact that the NADPH-stimulated metabolism of parathion by these two enzyme preparations is abolished by heating to 100° for 5 min and inhibited by incubation at pH values less than 7 and greater than 8·5, imply that cytochrome P-450-containing mixed-function oxidase enzyme systems are responsible for the metabolic activity seen. We were able to detect the presence of cytochrome P-450 in lung but not in rat brain microsomes. The failure to detect cytochrome P-450 in brain microsomes may have been because the concentration is too low to be detectable by the conventional method [16] used to quantitate this enzyme.

The apparent K_m and V_{max} values for sulfur binding to rat lung and rat brain microsomes were not significantly different from the analogous constants for paraoxon formation. Similar results were obtained using rat liver microsomes [12]. These and other data led to the conclusion that the sulfur bound to rat liver microsomes incubated with [^{35}S]parathion was that released in the mixed-function oxidase catalyzed metabolism of parathion to paraoxon [12]. The results of the present study imply that a similar conclusion is applicable to the binding of sulfur to rat lung and rat brain microsomes incubated with [^{35}S]parathion. The release and binding of sulfur to these microsomes may be the reason for the rapid departure of the rate of metabolism of parathion from linearity (Figs. 1 and 3). A previous study [12] has shown that incubation of parathion with rat liver microsomes in the presence of NADPH leads to a decrease in the concentration of cytochrome P-450 detectable as its carbon monoxide complex and a decrease in the rate of metabolism of benzphetamine compared to microsomes incubated with parathion in the absence of NADPH. Carbon disulfide (CS_2) is metabolized by rat liver microsomes to carbonyl sulfide (COS) [17]. As with parathion [12], the sulfur atom released becomes covalently bound to the microsomes. The mechanism of metabolism of CS_2 [17] is thought to be analogous to that proposed for the metabolism of parathion to paraoxon [18,19]. Additional studies with CS_2 [20] have shown that incubation of CS_2 with rat liver microsomes for varying periods of time in the presence of NADPH leads to a rapid (2 min or less) and progressive decrease in

the level of cytochrome P-450 detectable as its carbon monoxide complex and a comparable decrease in the ability of these microsomes to metabolize CS_2 and benzphetamine compared to controls incubated with NADPH but without CS_2 or CS_2 in the absence of NADPH. Incubation of rat liver microsomes with paraoxon [12] or COS [20] does not affect the level of cytochrome P-450 or metabolic activity toward benzphetamine. Thus, it appears that the binding of sulfur released in the metabolism of parathion to paraoxon or CS_2 to COS by rat liver microsomes rapidly inhibits the metabolism of these substrates. It appears quite possible that the rapid departure from linearity of the metabolism of parathion by rat lung and rat brain microsomes is also the result of the binding of the sulfur atom released in the metabolism of parathion to paraoxon, leading to an inactivation of the mixed-function oxidase enzyme system catalyzing the reaction.

Prior treatment of rats with compounds known to induce hepatic mixed-function oxidase activity toward parathion has been shown to decrease the toxicity of parathion in these animals [10,21–23]. In addition, male rats have a higher hepatic mixed-function oxidase activity toward parathion *in vitro* than females but are less susceptible to the acute toxic effects of parathion than are females. It has been postulated [10] that the decreased toxicity of parathion in phenobarbital-pretreated rats was a result of selective induction of the metabolism of parathion to its nontoxic metabolite diethyl phosphorothioic acid rather than to the anticholinesterase metabolite paraoxon. Thus, the amount of paraoxon formed in the liver of the phenobarbital-pretreated animals was less than in untreated animals, and the amount of paraoxon available to inhibit acetylcholinesterase in the lung and brain was decreased. This postulate was based on the finding that less diethyl phosphate, a product of the action of esterases on paraoxon, was excreted in the urine of phenobarbital compared to untreated rats. However, as has been pointed out previously [24], the actual concentration of paraoxon excreted in the urine was not measured in this study, and no attempt was made to determine how much of the paraoxon formed in the liver of these two treatment groups had been inactivated by reaction with proteins in the liver and serum. The work of Lauwerys and Murphy [25] and of Triolo *et al.* [26] has shown that "binding" and subsequent inactivation of paraoxon by proteins in the liver and plasma is a reaction important for the toxicity of this compound. The most probable explanation for this "binding" is the reaction of paraoxon with "serine active center" esterases and amidases in liver and serum leading to the formation of diethyl phosphate derivatives of these enzymes. This conclusion is supported by the finding that this "binding" is rapid and irreversible and is abolished by the heating of serum to 100° [26]. The examination of the metabolism of parathion by hepatic microsomes from phenobarbital-pretreated rats *in vitro* [4] also does not support the postulate of the selective induction of diethyl phosphorothioic acid formation *in vivo* [10]. These studies [4] showed an equal induction of the metabolism of parathion to paraoxon and to diethyl phosphorothioic acid. In addition, the apparent K_m value for the metabolism of parathion to diethyl phosphorothioic acid using hepatic microsomes from phenobarbital-pretreated rats was slightly but significantly greater than the apparent K_m value for parathion for formation of paraoxon [14]. Therefore, even at less than saturating parathion concentrations, as will be encountered *in vivo*, the rate of metabolism of parathion to diethyl phosphorothioic acid compared to paraoxon would not be expected to be greatly different than that seen *in vitro*.

An alternate hypothesis for the increased resistance to parathion of rats which have been pretreated with inducers of the hepatic mixed-function oxidase enzyme system is that there is an increase in the rate of the metabolism of parathion in the induced animals to both paraoxon and diethyl phosphorothioic acid, followed by hydrolysis of a portion of the paraoxon to diethyl phosphate by various esterases and a "binding" of the remainder of the paraoxon to specific proteins in the liver and serum [25,26]. Thus, any paraoxon formed in the liver is either hydrolyzed to inactive products or is bound to proteins in the serum and liver and never reaches sensitive tissues such as the lung or brain. The overall effect of this increased rate of hepatic metabolism of parathion in induced animals compared to noninduced would be lower concentrations of parathion at the active centers of enzymes in the lungs and brains capable of metabolizing parathion to paraoxon, which would, in turn, inhibit acetylcholinesterase in these organs. Inherent in this alternative hypothesis is the proposal that any paraoxon formed in the liver of non induced rats is also hydrolyzed or bound and never reaches the lung or brain. However, in the noninduced rats the rate of hepatic metabolism of parathion is less than in induced animals, and the concentration of parathion at the active centers of enzymes in the lungs and brains of these animals capable of metabolizing parathion to paraoxon is greater than in induced animals and the toxicity of a particular dose of parathion is greater.

The livers and plasma of rats have a large capacity for hydrolyzing paraoxon [10,27]. In fact, the rate of hydrolysis of paraoxon by these enzymes in the liver greatly exceeds the rate of formation of this metabolite by the mixed-function oxidase enzyme system in this organ [10]. However, the differences in the apparent K_m values for parathion for paraoxon formation in the liver (approximately 10^{-5} M) and for paraoxon for its hydrolysis by the various esterases (approximately 10^{-4} M) probably reduce the differential in the rate of formation and the rate of hydrolysis of paraoxon *in vivo*. However, when the combination of hydrolysis and binding to specific proteins in the liver and serum is considered, it appears unlikely that any paraoxon formed in the liver reaches the lung and brain.

Evidence supporting the view that the formation of paraoxon in the liver is not the mechanism by which parathion exerts its toxicity comes from the work of Jacobsen *et al.* [7]. These workers have found that parathion is five times more toxic to partially hepatectomized rats than to sham-operated controls. If the liver is the organ primarily responsible for the toxicity of parathion, one would expect that animals with a decreased hepatic capacity to metabolize par-

athion to paraoxon would be less rather than more susceptible to the toxic action of this compound.

Considering these various data, it appears likely that the mechanism of acute toxicity of parathion and, perhaps, other cholinergic phosphorothionate insecticides to mammals is the result of metabolism to their toxic oxygen analogues in the lung and brain. The toxic oxygen analogues, for example, paraoxon, are likely to be more persistent in these organs since no enzyme capable of the hydrolysis of paraoxon was detectable in these tissues [10]. The clear demonstration of the ability of mammalian lung and brain to catalyze the metabolism of parathion to paraoxon is essential to this hypothesis. This study and a previous study using rabbit lung microsomes [6] have, in fact, established that these organs are capable of this reaction.

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