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Studies on the Metabolism of Diethyl 4-Nitrophenyl Phosphorothionate (Parathion) *in vitro*

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1. The metabolism of the phosphorothionate parathion *in vitro* was examined by using [^{32}P]parathion and microsomes isolated from the livers of various animal species. 2. The major metabolic products of parathion in this system *in vitro* were identified as diethyl 4-nitrophenyl phosphate (paraoxon), diethyl hydrogen phosphate, diethyl hydrogen phosphorothionate and *p*-nitrophenol. 3. The reaction leading to the formation of diethyl hydrogen phosphorothionate and *p*-nitrophenol requires the same cofactors (NADPH and oxygen) required for metabolism of parathion to its active anti-acetylcholinesterase paraoxon. 4. The enzyme activity towards parathion per unit weight of liver is increased some 65–130% by pretreatment of male rats with phenobarbital and 3,4-benzopyrene. 5. The metabolism of parathion is inhibited by incubation in a nitrogen atmosphere and in an atmosphere containing carbon monoxide. Pure oxygen is also inhibitory. These results are discussed in terms of a deficiency of oxygen for maximal activity as well as the lability of some component of the system to oxidation.

The existence in various tissues of enzymes capable of metabolizing the dialkyl aryl phosphorothionates into active anti-acetylcholinesterases has been known for some time (Diggle & Gage, 1951*a*; Meyers, Mendel, Gersmann & Ketelaar, 1952; Gage, 1953; Metcalf & March, 1953; Kok & Walop, 1954; Davison, 1955; Murphy & DuBois, 1957; DuBois, Thrush & Murphy, 1957; Kubistova, 1959; O'Brien, 1959; Potter & O'Brien, 1964). Physical and chemical studies of the nature of this more toxic analogue of the dialkyl aryl phosphorothionates has shown it to be in most cases the corresponding phosphate (Gage, 1953; Brindley & Dahm, 1964; Nakatsugawa & Dahm, 1965). Exceptions are those dialkyl aryl phosphorothionates containing a thio ether group. Thus Benjamini, Metcalf & Fukuto (1959) showed that one of the active anti-acetylcholinesterases resulting from the metabolism of diethyl 4-methylsulphinylphenyl phosphorothionate (Bayer 25141) in insects was the corresponding sulphone. Various other studies carried out *in vivo* and *in vitro* have shown that the dialkyl aryl phosphates and phosphorothionates are metabolized by various tissues into monoalkyl aryl phosphates, monoalkyl aryl phosphorothionates and dialkyl and monoalkyl phosphates and phosphorothionates plus the corresponding phenols (Aldridge, 1953; Plapp & Casida, 1958; Brady & Arthur, 1961; Krueger & Casida, 1961; Matsumura & Hogendijk, 1964).

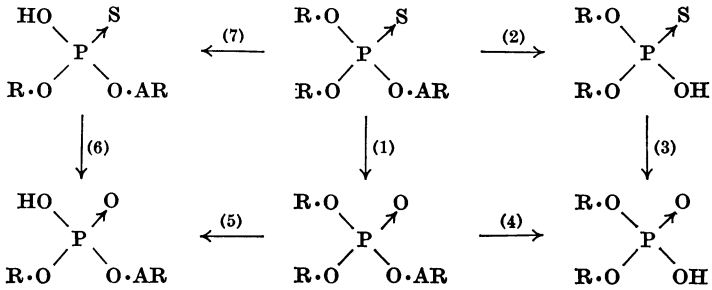
The metabolism of the dialkyl aryl phosphorothionates may therefore be represented as shown in Scheme 1 (Plapp & Casida, 1958).

Reaction (1), the metabolism of the phosphorothionate to its corresponding phosphate, has already been discussed. The enzymes that carry out this reaction are located in the microsomal fraction or, in insects, in the particulate fraction equivalent to microsomes. This reaction requires as cofactors NADPH and oxygen.

The nature of the enzymes and cofactors involved in reactions (2), (3), (5), (6) and (7) has not yet been elucidated.

Reaction (4), the metabolism of the dialkyl aryl phosphate to the corresponding dialkyl phosphate and phenol, has been shown to be located in various tissues (Aldridge, 1953; Main, 1960; Augustinsson & Heimburger, 1954) and in various parts of the cell (Neal & DuBois, 1965). The enzyme(s) that catalyses this reaction is an esterase (Aldridge, 1953).

The present paper describes an investigation of the metabolism *in vitro* of the dialkyl aryl phosphorothionate parathion, with particular attention to the nature of the reaction leading to the formation of diethyl hydrogen phosphorothionate and the



Scheme 1. Metabolism of dialkyl aryl phosphorothionates. R, alkyl; Ar, aryl.

quantitative relationship between the production of this metabolite and the production of the active anti-acetylcholinesterase paraoxon.

MATERIALS AND METHODS

Isolation of microsomes. Adult male and female Sprague–Dawley rats (100–200g.), adult male mice and adult male guinea pigs (500–600g.) were used in these experiments. The animals were fed on a stock diet *ad libitum* and kept in air-conditioned quarters under artificial lighting.

The animals were killed by decapitation and the tissues were quickly removed, weighed and homogenized in 4·0vol. of iso-osmotic KCl (1·15%, w/v) containing nicotinamide (0·25%). Microsomes were prepared by first centrifuging the whole homogenate at 9000*g* for 20min. in a refrigerated Servall centrifuge with a SS–34 rotor. The supernatant from this step was centrifuged at 105000*g* for 90min. in a Spinco model L preparative centrifuge (40 rotor). The supernatant from this centrifugation was designated the soluble fraction. The microsomal pellet was quickly frozen in an acetone–solid CO_2 bath and stored at −15° until ready for use. At this temperature the microsomes maintained essentially full activity towards parathion for as long as 3 weeks. All fractionation procedures were carried out at 4° or below. When the microsomes were used in an experiment they were added to the reaction mixture as a suspension in cold distilled water.

The activity of various preparations of microsomes varied, and there was some day-to-day variation of activity in the same preparation of microsomes. Comparisons of activities of various changes in the incubation conditions were therefore always made in simultaneous experiments.

Synthesis of [^{32}P]*parathion.* [^{32}P]Parathion (diethyl 4-nitrophenyl [^{32}P]phosphorothionate) was synthesized from 20mc of carrier-free $NaH_2{}^{32}PO_4$ by first exchanging with P_2S_5 (1·3m-moles) according to the method of Casida (1958). Ethanol (5·2m-moles) dissolved in 1ml. of CCl_4 was then added dropwise over 30min. to a stirred suspension of the P_2S_5 in 2ml. of CCl_4 heated at 60°. After addition of the ethanol the reaction mixture was stirred at 60° for a further 90min., after which time it was heated to boiling and refluxed for 2hr. The desired product of this reaction (diethyl hydrogen phosphorodithionate) was not isolated. Instead, the reaction mixture was cooled to 20° and a solution of chlorine dissolved in CCl_4 was added dropwise until the greenish-yellow colour of chlorine no longer disappeared as a drop of the chlorine in CCl_4 was added to the reaction mixture. The addition of the chlorine took place over approx. 15min. The desired product of this reaction (diethyl phosphorochloridothionate) was also not isolated. Instead, the reaction mixture was extracted three times with 5ml. portions of 5% (w/v) Na_2CO_3. The organic layer was dried over anhydrous Na_2SO_4 and added dropwise to a refluxing solution of 1·25m-moles of the sodium salt of *p*-nitrophenol in 7ml. of acetone. The reaction mixture was refluxed with stirring for 5hr. The reaction mixture was then extracted with 10ml. portions of 5% (w/v) Na_2CO_3 until the yellow colour of *p*-nitrophenol no longer appeared in the aqueous layer. The organic layer was then washed twice with distilled water and dried over anhydrous Na_2SO_4. The range of specific activities and yields for parathion were 3·5–5·0mc/m-mole and 15–19% respectively. Samples of parathion, paraoxon (diethyl 4-nitrophenyl phosphate), diethyl hydrogen phosphate and diethyl hydrogen phosphorothionate were kindly supplied by the Monsanto Co., St Louis, Mo., U.S.A., and the American Cyanamide Co., Princeton, N.J., U.S.A.

The synthesized [^{32}P]parathion was purified by first applying a hexane solution of the synthesized compound to the top of a 2·5cm. × 45·0cm. column of Woelm alumina [acid (anionotropic); Brockmann grade V (15% of water)]. The column was first washed with 200ml. of hexane and [^{32}P]parathion was eluted from the column with 200ml. of hexane–benzene (1:1, v/v). The product was further purified by ascending one-dimensional thin-layer chromatography by applying approx. 25mg. of the impure product as a streak on a line 2cm. from the bottom of an 8in. × 8in. thin-layer plate coated with 1mm.-thick layer of silica gel G (Brinkmann Instruments Inc., Westbury, N.Y., U.S.A.). Approx. 300μg. of authentic parathion was spotted at the origin at both ends of the streak of the impure product and the chromatogram developed with hexane–acetone (4:1, v/v). The parathion was located under u.v. light and the band corresponding to parathion outlined on the thin-layer plate by using the sharp end of a small spatula. The silica gel to which the [^{32}P]parathion was adsorbed was scraped from the plate, packed into a small glass column (1·0cm. × 10cm.), and the parathion eluted from the silica gel with two 10ml. portions of methanol, followed by one 10ml. portion of chloroform–methanol (1:1, v/v). The identity of the synthesized parathion was proved by comparison of the infrared spectrum of a solution of the synthesized product in $CHCl_3$ with that of authentic parathion.

Incubation conditions. Except where noted, the incubation system used in these studies is as follows: phosphate buffer (pH8·0), 30μmoles; NADP, 1·3μmoles; glucose

6-phosphate, 5·6 μmoles; parathion, 0·35 μmole (added as a 13·6mM solution in ethanol); microsomes equivalent to 100mg. of liver. Sufficient distilled water was added to make a final volume of 2·0ml. The mixture was incubated with shaking at 37° in 20ml. beakers open to the air. Except where noted in the text, the incubation period was 1hr.

Metabolism studies: isolation and estimation of metabolites. In the experiments designed to determine the metabolites of parathion (exclusive of *p*-nitrophenol) the incubations were terminated by the addition of 3·0ml. of cold acetone and the reaction mixtures kept at −20° for 1hr. The precipitated proteins were removed by centrifugation and the supernatants adjusted to pH3–4 with 1·0N-HCl. The reason for lowering the pH of the supernatant fraction is to prevent non-enzymic breakdown of parathion in subsequent steps in the isolation of the metabolites. The volume of the supernatant was accurately measured and a 50 μl. portion of each supernatant spotted on each of two separate 8in. × 8in. glass plates, which were coated with 250 μ-thick layers of silica gel G (Brinkmann Instruments Inc.). A 50 μl. portion of a combined substrate and tissue blank was also spotted on each of the two thin-layer plates. The blank was prepared by incubation of microsomes and cofactors without substrate, addition of acetone and then addition of substrate. The substrate was incubated in a small volume of buffer for the same period of time as the microsomes plus cofactors. The amount of non-enzymic hydrolysis of parathion to *p*-nitrophenol and diethyl hydrogen phosphorothionate during the incubation averaged about 0·5–1% of the amounts of these metabolites formed enzymically. Portions of as many as nine different supernatant fractions were spotted on the same plate. It is important not to use hot air as an aid in spotting the supernatant fractions on the thin-layer plates since this leads to volatilization of some of the reaction products. After spotting, the plates were subjected to ascending chromatography in filter-paper-lined glass tanks. Two different solvent systems were used. One plate was eluted with methanol–chloroform–aq. 10% (w/v) NH_3 (24:75:3·5, by vol.) (solvent system A), and the other with hexane–chloroform–methanol (7:2:1, by vol.) (solvent system B). After the solvent front had migrated 10cm. past the origin, the plates were removed from the tanks, dried, marked in the four corners with radioactive ink and placed in contact with 8in. × 10in. 'no screen' X-ray film (Eastman Kodak Co., Rochester, N.Y., U.S.A.). The plates were left in contact with the film for 12–24hr. depending on the specific activity of the [^{32}P]parathion used in the incubations. The radioactive areas on the thin-layer plates were isolated and their radioactivity was determined quantitatively in the following manner. First, the developed film was clipped on to the thin-layer plate in the same position that existed during development of the film, as indicated by the position of the spots on the film that correspond to the position of the spots of radioactive ink on the thin-layer plate. The plate with the attached film was then placed on an X-ray film viewer with the uncoated side of the glass plate upward. The dark spots on the film corresponding to areas of radioactivity on the plate are thus easily located through the layer of silica gel. The dark areas on the film were transcribed on to the back of the thin-layer plate with a felt-tipped pen. The film was then removed from the thin-layer plates and the plates were placed on a white background with the silica-gel-layered side upward. The silica gel corresponding to each of the inked-in areas on the back of the plate was then removed, placed in counting vials and counted in a Packard model 3000 liquid-scintillation spectrometer. The scintillation fluid consisted of 100g. of naphthalene, 7g. of 2,5-diphenyloxazole and 0·3g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (Packard Instrument Co., Downers Grove, Ill., U.S.A.) dissolved in 500ml. of 1,4-dioxan and made up to 1l. with 1,4-dioxan. Use of the internal recovery technique indicated there was no quenching due to the silica gel present in the counting vials. The use of Cab-O-Sil (Packard Instrument Co.) to disperse the silica gel evenly throughout the scintillation fluid slightly decreased the counting efficiency as compared with counting without the use of Cab-O-Sil.

The non-radioactive phosphorothionates were located on the thin-layer plates by spraying with 0·5% $PdCl_2$ in 0·1N-HCl. The phosphates were located by spraying the developed thin-layer plates with the phosphorus-sensitive spray described by Hanes & Isherwood (1949).

RESULTS

The reaction mixtures resulting from the incubation of [^{32}P]parathion with male rat liver microsomes were examined for metabolic breakdown products other than *p*-nitrophenol by thin-layer chromatography. It had already been shown (Neal & DuBois, 1965) that *p*-nitrophenol was a product of the action of microsomes on parathion. When a portion of a deproteinized reaction mixture was spotted on a thin-layer plate and the plate eluted with solvent system A, the subsequent radioautographs revealed the presence of five different areas of radioactivity (Table 1). The average R_F values of these areas of radioactivity, as measured to the centre of the area of exposure on the film, were 0·05, 0·24, 0·42, 0·59 and 1·00.

Table 1. *Thin-layer chromatography of parathion metabolites*

The results shown are the averages (with ranges in parentheses) of R_F values of the metabolites of parathion formed during incubation of parathion with male rat liver microsomes (see the Materials and Methods section for incubation conditions and isolation procedures). Solvent system A consisted of methanol–chloroform–aq. 10% (w/v) NH_3 (24:75:3·5, by vol.). Solvent system B consisted of hexane–chloroform–methanol (7:2:1, by vol.). These values represent the average of 16 different radioautographs.

R_F value	
Solvent system A	Solvent system B
0·05 (0·02–0·10)	0·05 (0·02–0·08)
0·24 (0·10–0·44)	0·58 (0·45–0·70)
0·42 (0·24–0·65)	0·62 (0·50–0·77)
0·59 (0·41–0·75)	1·00
1·00	

By co-chromatography with known compounds the compounds with average R_F values 0·24, 0·42 and 1·00 were tentatively identified as diethyl hydrogen phosphate, diethyl hydrogen phosphorothionate and a combination of paraoxon and parathion respectively. The compounds with R_F values 0·05 and 0·59 each represented less than 1% of the total radioactivity exclusive of unmetabolized parathion. Attempts to identify these two compounds have not yet been made. The metabolites with average R_F values 0·24 and 0·42 were proved to be diethyl hydrogen phosphate and diethyl hydrogen phosphorothionate by comparison of the infrared spectra of these two metabolites with authentic compounds by using the potassium bromide micro-pellet technique.

When the thin-layer plates were eluted with solvent system B, an examination of the subsequent radioautographs revealed the presence of four areas of radioactivity with average R_F values 0·05, 0·58, 0·62 and 1·00. The compound with average R_F 0·05 was obviously made up of more than one compound, as revealed by the striped appearance of this area of radioactivity. Subsequent co-chromatography with known compounds and with the metabolites obtained from a thin-layer plate eluted with solvent system A revealed that this radioactive area contained diethyl hydrogen phosphate, diethyl hydrogen phosphorothionate and the two unknown compounds with average R_F values 0·05 and 0·59 on a thin-layer plate that had been eluted with solvent system A. The compound with average R_F 0·58 represents less than 1% of the total activity exclusive of unmetabolized parathion and has not yet been identified. The compound with average R_F 0·62 was identified as paraoxon by co-chromatography with authentic paraoxon in three different solvent systems. The compound with R_F 1·00 was identified as unchanged parathion.

The appearance of diethyl hydrogen phosphate, diethyl hydrogen phosphorothionate and paraoxon

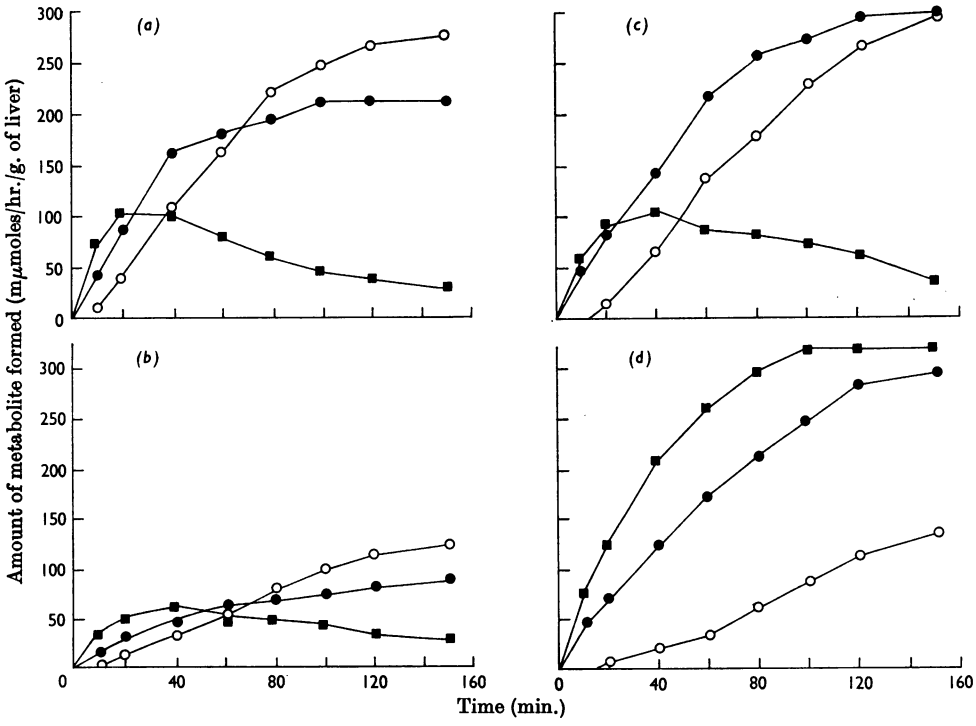


Fig. 1. Microsomes isolated from the pooled livers of four each adult male rats (*a*), adult female rats (*b*), adult male mice (*c*) and adult male guinea pigs (*d*) were incubated with parathion as described in the Materials and Methods section, except that the reactions were terminated at different times by addition of cold acetone. The amounts of metabolites formed were measured as described in the Materials and Methods section. The incubations with the various groups of animals were carried out on successive days. In each experiment the microsomes were used within 30 min. after isolation to minimize the loss in the activity of some of the microsomal enzymes that is seen on aging of the microsomes. ■, Paraoxon; ●, diethyl hydrogen phosphorothionate; ○, diethyl hydrogen phosphate.

with time in various animal species is shown in Fig. 1. An examination of Fig. 1 reveals some quantitative differences in the metabolism of parathion by the various animal species. For example, the adult female rat displays a generally lower overall activity towards parathion than any other animal examined. The other major quantitative difference is the greater accumulation of paraoxon along with a concomitant lower accumulation of diethyl hydrogen phosphate in the adult male guinea pig as compared with the other animals examined. Similar differences were also seen in a study of the metabolism of ethyl 4-nitrophenyl phenylphosphonothionate (EPN) and its oxygen analogue (Neal & DuBois, 1965). In this latter study the appearance of *p*-nitrophenol was used as the measure of the level of metabolic activity.

In all animals investigated the metabolism of parathion in the present system *in vitro* seemed to follow a pattern of an initial rapid formation of diethyl hydrogen phosphorothionate and paraoxon during the first 20min. With the exception of the guinea pig, the amount of paraoxon in the reaction medium then levels off, stays constant for approx. 20min. and then decreases. At the same time as the levelling off in the concentration of paraoxon occurs, there is a rapid increase in the concentration of diethyl hydrogen phosphate in the reaction medium. The concentration of diethyl hydrogen phosphorothionate continues to increase for about 90min., after which time the amount in the incubation medium begins to level off. The metabolism of parathion in this system *in vitro* has essentially stopped after 2hr. incubation.

The data in Fig. 1 seem to indicate that paraoxon formation ceases after about 20min. incubation. However, with the exception of the guinea pig, paraoxon continues to be formed from parathion by the action of liver microsomes after 20min. incubation, but is broken down to diethyl hydrogen phosphate and *p*-nitrophenol at a rate that prevents further increase in the concentration of this compound in the incubation mixtures. The further production of paraoxon from parathion after 20min. incubation can be demonstrated (Fig. 2) by adding EDTA to the incubation mixture. EDTA inhibits an enzyme (or enzymes) in microsomes that metabolizes paraoxon to diethyl hydrogen phosphate and *p*-nitrophenol. This enzyme is probably similar to the esterase enzyme described by Aldridge (1953). A comparison of Figs. 1 and 2 reveals that inclusion of EDTA in the incubation medium containing male rat liver microsomes causes the concentration of paraoxon to continue to increase at a rate similar to that of diethyl hydrogen phosphorothionate and that the formation of diethyl hydrogen phosphate is

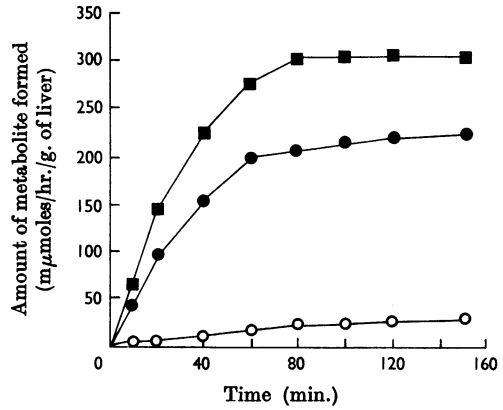


Fig. 2. Microsomes isolated from the same pooled livers of adult male rats described in Fig. 1 were incubated with parathion as described in the Materials and Methods section, except that EDTA (3mM) was added at the beginning of the incubation and the reactions were terminated at different times by addition of cold acetone. The metabolites formed were isolated and measured as described in the Materials and Methods section. ■, Paraoxon; ●, diethyl hydrogen phosphorothionate; ○, diethyl hydrogen phosphate.

strongly inhibited. Barium salts and, to a smaller extent, manganous salts also inhibit the metabolism of paraoxon. The effects of Ba^{2+}, EDTA and Mn^{2+} are partially reversed by addition of equimolar amounts of Ca^{2+} to the incubation media containing these inhibitors.

The amount of *p*-nitrophenol formed during the incubations consistently exceeds the sum of the diethyl hydrogen phosphorothionate and diethyl hydrogen phosphate formed by about 15%. This discrepancy may be a result of loss of some of the phosphate compounds during isolation owing to volatility.

In the absence of the NADPH-generating system there was no metabolism of parathion detectable either by the appearance of radioactive metabolites or of *p*-nitrophenol.

Approx. 50μg. of the radioactive metabolites diethyl hydrogen phosphate and diethyl hydrogen phosphorothionate were isolated from a series of thin-layer chromatograms and incubated separately with microsomes in the same manner described for parathion. An examination of the incubation medium failed to reveal any further metabolism of these two compounds.

Although the formation of paraoxon from parathion had been reported in tissues other than liver (Kubistova, 1959), it was decided to examine the quantities of the various metabolites of parathion formed in these tissues in comparison

with liver. Table 2 shows the results of this study. This experiment was carried out with the supernatant fractions remaining after sedimenting the mitochondria, nuclei and cell debris (9000*g* supernatant). The 9000*g* supernatant fraction from rat liver exhibits some two- to three-fold more enzymic activity towards parathion than microsomes isolated from the same homogenate. Investigation has shown that this increased activity is not due to a greater availability of NADPH but rather it is apparently a result of an increased stability of the microsomal enzymes that metabolize parathion.

An examination of Table 2 reveals there is metabolic activity towards parathion in tissues other than liver, but the activity is considerably less than in liver. The results shown were obtained with the tissues of a single adult male rat, but are representative of results obtained with other single animals.

Table 2. *Comparison of the enzyme activity towards parathion in various tissues*

This experiment was conducted with the microsomes +supernatant fraction (9000*g* supernatant) from the various tissues. The procedures for incubation and for isolation and measurement of the quantities of metabolites are given in the Materials and Methods section.

	Amount of metabolite formed (mμmoles/hr./g. of tissue)		
Tissue	Diethyl hydrogen phosphorothionate	Diethyl hydrogen phosphate	Paraoxon
Lung	9	3	13
Brain	9	2	4
Kidney	13	3	32
Liver	417	353	98

The effect of pretreatment of male rats with phenobarbital and 3,4-benzopyrene on the activity of the microsomal enzymes that metabolize parathion is shown in Table 3. It is apparent that pretreatment with these two compounds considerably increased the activity, per unit weight of liver, of the enzymes that metabolize parathion. The effect of pretreatment with these compounds on the level of activity towards parathion in tissues other than liver was also examined. In contrast with the enzymes in the liver, no significant increase in the activity of the enzymes in lung, kidney and brain, as compared with the untreated animals, could be detected.

That the enzymes that metabolize parathion can be induced by phenobarbital and 3,4-benzopyrene and require NADPH for activity strongly suggests that the initial reactions that parathion undergoes are catalysed by the same coupled microsomal enzyme systems, the so-called 'mixed-function' oxidases, that carry out various hydroxylation, sulphoxidation and demethylation reactions. To test this relationship further, the effect of carbon monoxide and various other gases and mixtures of gases on the metabolism of parathion was examined (Table 4). The incubations were conducted for two different time-periods. The apparently greater activity in the 15min. incubation, as compared with 60min. incubation, is due to loss of linearity of the production of metabolites in this system *in vitro* after approx. 20min. incubation. An examination of the results obtained in these experiments reveals a rather complicated picture. After 15min. incubation in a nitrogen atmosphere the amounts

Table 3. *Enzyme activity towards parathion in livers of untreated, phenobarbital-treated and 3,4-benzopyrene-treated adult male rats*

Microsomes isolated from pooled livers of four rats in each of the treatment groups were incubated with parathion as described in the Materials and Methods section. The phenobarbital-treated animals received a dose (80mg./kg.) of phenobarbital dissolved in 0·9% NaCl by intraperitoneal injection daily for 4 days and were killed 24hr. after the last injection. The 3,4-benzopyrene-treated animals received a dose (20mg./kg.) of 3,4-benzopyrene dissolved in corn oil by intraperitoneal injection daily for 3 days and were killed 24hr. after the last injection. The animals used in this experiment were from a single shipment and were of approximately the same age (±2 days). All animals used in this experiment were killed on the same day. The results are given as the means ±S.D. of four separate incubations.

	Amount of metabolite formed (mμmoles/hr./g. of liver)		
Treatment	Diethyl hydrogen phosphorothionate	Diethyl hydrogen phosphate	Paraoxon
None	165±10	187±8	86±3
Phenobarbital	283±13	362±3	93±2
3,4-Benzopyrene	241±8	486±9	141±6

Table 4. *Effect of various gases and mixtures of gases on the metabolism of parathion*

The buffer, substrate, cofactors and microsomes, in the amounts indicated in the Materials and Methods section, were pipetted into 25ml. Erlenmeyer flasks immersed in an ice bath. All flasks were stoppered with a rubber stopper containing a 1mm. hole in the centre, and flushed vigorously with N_2 for 1min. by using a glass capillary inserted through the small hole in the stopper. The flasks were then sealed by inserting a small glass rod in the hole in the stopper as the capillary was withdrawn. Those flasks incubated in a N_2 atmosphere remained sealed. The small glass rod was removed from the hole in the stopper of those flasks incubated in an air atmosphere and the flasks were allowed to equilibrate with the air during the incubation. Those flasks incubated in an atmosphere of N_2+O_2 (98:2), N_2+CO+O_2 (56:42:2), N_2+CO (75:25), O_2 and CO were flushed for 1min. with the appropriate gas or mixtures of gases and the flasks resealed by reinsertion of the glass rod as the capillary was withdrawn. The flasks were incubated in the dark for the times indicated and the metabolites isolated and measured as described in the Materials and Methods section. The values given are the means ± S.D. of three separate determinations carried out simultaneously with microsomes isolated from the same sample of pooled male rat livers.

Incubation atmosphere	Amount of metabolite formed (mμmoles/hr./g. of liver): Diethyl hydrogen phosphorothionate	Paraoxon + diethyl hydrogen phosphate
Incubation time 60min.		
Air	191±2	386±7
N_2	169±4	424±6
O_2	139±7	249±5
CO	0	0
N_2+O_2 (98:2)	188±10	338±6
N_2+CO+O_2 (56:42:2)	176±2	289±8
N_2+CO (75:25)	104±10	205±9
Incubation time 15min.		
Air	372±24	920±32
N_2	96±12	450±46
O_2	280±28	645±21
CO	0	0
N_2+O_2 (98:2)	384±16	882±18
N_2+CO+O_2 (56:42:2)	336±8	719±13

of both diethyl hydrogen phosphorothionate and of paraoxon plus diethyl hydrogen phosphate formed are depressed in comparison with the results of incubation in air. After 60min. incubation, however, although the formation of diethyl hydrogen phosphorothionate is still inhibited ($P < 0.01$) in the nitrogen atmosphere, the formation of paraoxon plus diethyl hydrogen phosphate is significantly increased ($P < 0.01$) over the amount formed in an air atmosphere. These results could be explained on the basis of an increased stability in a nitrogen atmosphere of some component (or components) in the enzyme system that metabolizes parathion to paraoxon balanced against an insufficient amount of oxygen for maximal enzyme activity. The results with pure oxygen seem to indicate that some component in the system or systems is susceptible to an oxidation that leads to a decreased enzymic activity. That this susceptible group may be a thiol group is supported by a partial reversal of the oxygen inhibition in the presence of mercaptoethanol. Mercaptoethanol also stimulates the formation of both diethyl hydrogen phosphorothionate and paraoxon in an air and, to a smaller extent, in a nitrogen atmosphere. The stimulating effect of nitrogen atmosphere on paraoxon formation during the 60min. incubation may therefore be due to partial protection of a thiol group from oxidation. The results obtained with incubation in a nitrogen + oxygen (98:2) atmosphere do not differ significantly from the results obtained by incubation in an air atmosphere when the incubation time is 15min., but there is a significant decrease ($P < 0.01$) in the amount of paraoxon formed during a 60min. incubation in the nitrogen + oxygen (98:2) atmosphere as compared with that formed in an air atmosphere. The reason for this is also not known, but may be due to insufficient oxygen for maximal activity that is only partially compensated for by an increased stability of the enzyme system due to the presence of a high percentage of nitrogen in the incubation atmosphere. The metabolism of parathion seems to be considerably less susceptible to carbon monoxide than other systems reported. Thus Cooper, Levin, Narasimhulu, Rosenthal & Estabrook (1965) reported a 50% inhibition of the demethylation of codeine in the presence of a carbon monoxide/oxygen ratio 1·0. Kratz & Staudinger (1965) reported a 90% inhibition of the hydroxylation of coumarin in the presence of a carbon monoxide/oxygen ratio 2·0. There can be little doubt that carbon monoxide is inhibitory in this system, however, since an incubation atmosphere consisting of nitrogen + carbon monoxide + oxygen (56:42:2) significantly inhibits ($P < 0.01$–0·05) the metabolism of parathion during both 15min. and 60min. incubation. In addition, a nitrogen + carbon monoxide (75:25) incubation atmosphere strongly inhibits and pure carbon monoxide completely inhibits the metabolism of parathion.

The results of the experiments described in Table 4 seem to indicate the K_m for oxygen in this parathion-metabolizing system is very low and that sufficient oxygen may remain dissolved in solution or bound to the microsomes despite vigorous flushing with nitrogen to give significant activity in a nitrogen atmosphere.

DISCUSSION

The results of this investigation indicate that parathion is metabolized to paraoxon by an enzyme system that resembles the so-called 'mixed-function' oxidase systems that carry out various hydroxylation, sulphoxidation and demethylation reactions. The paraoxon is in turn metabolized to diethyl hydrogen phosphate and *p*-nitrophenol. Concurrent with the formation of paraoxon, a portion of the parathion is metabolized to diethyl hydrogen phosphorothionate and *p*-nitrophenol by a reaction that also requires NADPH and molecular oxygen.

These results are similar to those described by Scaife & Campbell (1959) for the metabolism of Amiton (*S*-2-diethylaminoethyl *OO*-diethyl phosphorothiolate). However, these workers reported little or no enzymic activity towards Amiton in tissues other than liver. In addition, NAD^+ or NADH, rather than NADPH, was the active cofactor in their system.

Three possible explanations for the concurrent formation of paraoxon and diethyl hydrogen phosphorothionate from parathion are as follows. (1) The parathion becomes attached to a single site on an enzyme in such a way that both the sulphur and *p*-nitrophenol groups are free to leave. In a concerted reaction in which an incoming group is an enzyme-bound hydroxyl group or hydroxyl radical, the sulphur may leave, giving rise to paraoxon, or alternatively the *p*-nitrophenol may be the leaving group, giving rise to diethyl hydrogen phosphorothionate. (2) There are two different binding sites for parathion on the same enzyme. At one site parathion may be bound in such a way as to facilitate the removal of sulphur by a concerted mechanism, and at the other the *p*-nitrophenol group is the leaving group, giving rise to paraoxon and diethyl hydrogen phosphorothionate respectively. (3) There may be two separate enzymes or enzyme systems involved in the metabolism of parathion, one that forms paraoxon from parathion, and another that metabolizes the parent compound to diethyl hydrogen phosphorothionate and *p*-nitrophenol. Results obtained with bivalent cations and some preliminary results with various inhibitors favour proposal (2) or (3). The bivalent cations Mg^{2+} and Ca^{2+} significantly increase the metabolism of parathion to diethyl hydrogen phosphorothionate in the present system *in vitro*, but have no statistically significant effect on the conversion of parathion into paraoxon, with the sum of the paraoxon and diethyl hydrogen phosphate being considered as the measure of the total conversion of parathion into paraoxon. Preliminary work with inhibitors indicates a differential inhibition of the formation of paraoxon and of diethyl hydrogen phosphorothionate from parathion; this would also support the presence of two binding sites or two separate enzymes or enzyme systems. The presence of two sites on a single enzyme catalysing different reactions has little or no precedence. Therefore the best explanation for the metabolic and preliminary inhibitor data is the presence of two separate enzyme systems.

Additional evidence for two separate enzymes or enzyme systems is that, although pretreatment with 3,4-benzopyrene stimulates the metabolism of parathion to both paraoxon and diethyl hydrogen phosphorothionate, the production of paraoxon is stimulated to a greater degree. The data in Table 3 show that diethyl hydrogen phosphorothionate production from parathion in 3,4-benzopyrene-treated animals is stimulated some 45% as compared with untreated animals, whereas the production of paraoxon is stimulated some 130%. In the phenobarbital-treated animals about equal stimulation of the metabolism of parathion to diethyl hydrogen phosphorothionate and paraoxon was seen (72 and 66% respectively). In addition, incubation of liver microsomes with parathion for 1hr. in a nitrogen atmosphere leads to a decrease in the metabolism of parathion to diethyl hydrogen phosphorothionate, but an increase in the production of paraoxon in comparison with the results of incubation for the same period of time in air (see Table 4).

It has generally been assumed that the toxic effect of the phosphorothionates *in vivo* is a result of metabolism to their oxygen analogues in the liver and migration of these oxygen analogues to the nerve endings where they inhibit the enzyme acetylcholinesterase, leading to acetylcholine accumulation with a subsequent disruption of nerve function (O'Brien, 1960). However, *in vitro* the male rat liver converts parathion into its toxic oxygen analogue at a much faster rate than the liver of the female rat, yet is much more resistant *in vivo* to the toxic effects of this compound. In addition, pretreatment of rats with phenobarbital has been shown in the present study to increase the rate of conversion of parathion into its toxic oxygen analogue paraoxon. Yet pretreatment of rats with phenobarbital decreases rather than increases the toxicity of parathion administered by intraperitoneal injection *in vivo* (R. A. Neal, unpublished work). Ball, Sinclair, Crevier & Kay (1954) and Main (1956) have reported that pretreatment of male and female rats with aldrin, a chlorinated hydrocarbon that stimulates drug metabolism in the rat (Cram, Juchau & Fouts, 1965), decreases both the oral and intravenous toxicity of parathion.

A decreased toxicity of ethyl 4-nitrophenyl phenylphosphonothionate (EPN) administered by

intraperitoneal injection *in vivo* on pretreatment of rats with phenobarbital was also observed by DuBois & Kinoshita (1966). These results seem to indicate that any of the paraoxon formed in the liver is probably hydrolysed to diethyl hydrogen phosphate and *p*-nitrophenol before it reaches the nerve endings and that the reaction responsible for the toxicity of parathion and perhaps other phosphorothionates is the extrahepatic metabolism of the phosphorothionate to its toxic oxygen analogue. The fact that hepatectomy does not decrease the susceptibility of rats to parathion (Diggle & Gage, 1951*b*) tends to support this proposal. Therefore it appears that metabolism of parathion and perhaps other phosphorothionates of similar structure in the liver may actually lead to a decrease in the toxicity of a particular exposure to the compound by decreasing the circulating concentration or total amount of the phosphorothionate in the animal. A similar hypothesis has been advanced by Main (1956). On the other hand, Gaines, Hayes & Linder (1966) have published results that may be in conflict with this hypothesis. These workers reported a greater toxicity for parathion when injected directly into the hepatic portal circulation of adult female rats than when injected into the femoral vein. All of the parathion injected directly into the hepatic portal circulation would pass through the liver before distribution to the rest of the body, whereas only a portion (approx. 27%) of parathion injected into the femoral vein would first pass through the liver. It would thus appear that whether the liver functions to decrease the toxicity of a particular exposure to parathion may depend on the rate of administration, the route of exposure and perhaps the sex of the animal in the case of rats.

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REFERENCES

Aldridge, W. N. (1953). *Biochem. J.* **53**, 117.
Augustinsson, K. B. & Heimburger, G. (1954). *Acta chem. scand.* **8**, 1533.
Ball, W. L., Sinclair, J. W., Crevier, M. & Kay, K. (1954). *Canad. J. Biochem. Physiol.* **32**, 440.
Benjamini, E., Metcalf, R. L. & Fukuto, T. R. (1959). *J. econ. Ent.* **52**, 94.
Brady, U. E., jun. & Arthur, B. W. (1961). *J. econ. Ent.* **54**, 1232.
Brindley, W. A. & Dahm, P. A. (1964). *J. econ. Ent.* **57**, 47.
Casida, J. E. (1958). *Acta chem. scand.* **12**, 1691.
Cooper, D. Y., Levin, S., Narasimhulu, S., Rosenthal, O. & Estabrook, R. W. (1965). *Science*, **147**, 400.
Cram, R. L., Juchau, M. R. & Fouts, J. R. (1965). *J. Lab. clin. Invest.* **66**, 906.
Davison, A. N. (1955). *Biochem. J.* **61**, 203.
Diggle, W. M. & Gage, J. C. (1951*a*). *Biochem. J.* **49**, 491.
Diggle, W. M. & Gage, J. C. (1951*b*). *Nature, Lond.*, **168**, 998.
DuBois, K. P. & Kinoshita, F. (1966). *Proc. Soc. exp. Biol., N.Y.*, **121**, 59.
DuBois, K. P., Thrush, D. R. & Murphy, S. D. (1957). *J. Pharmacol.* **119**, 208.
Gage, J. C. (1953). *Biochem. J.* **54**, 426.
Gaines, T. B., Hayes, W. J., jun. & Linder, R. E. (1966). *Nature, Lond.*, **209**, 88.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Kok, G. C. & Walop, J. N. (1954). *Biochim. biophys. Acta*, **13**, 510.
Kratz, F. & Staudinger, H. (1965). *Hoppe-Seyl. Z.* **343**, 27.
Krueger, H. R. & Casida, J. E. (1961). *J. econ. Ent.* **54**, 239.
Kubistova, J. (1959). *Arch. int. Pharmacodyn.* **118**, 308.
Main, A. R. (1956). *Canad. J. Biochem. Physiol.* **34**, 197.
Main, A. R. (1960). *Biochem. J.* **74**, 10.
Matsumura, F. & Hogendijk, C. J. (1964). *J. Agric. Fd. Chem.* **12**, 447.
Metcalf, R. L. & March, R. B. (1953). *Ann. Amer. Ent. Soc.* **46**, 63.
Meyers, D. K., Mendel, B., Gersmann, H. R. & Ketelaar, J. A. A. (1952). *Nature, Lond.*, **170**, 805.
Murphy, S. D. & DuBois, K. P. (1957). *J. Pharmacol.* **119**, 572.
Nakatsugawa, T. & Dahm, P. A. (1965). *J. econ. Ent.* **58**, 500.
Neal, R. A. & DuBois, K. P. (1965). *J. Pharmacol.* **148**, 185.
O'Brien, R. D. (1959). *Nature, Lond.*, **183**, 121.
O'Brien, R. D. (1960). *Toxic Phosphorus Esters*, p. 209. New York and London: Academic Press Inc.
Plapp, F. W. & Casida, J. E. (1958). *J. econ. Ent.* **51**, 800.
Potter, J. L. & O'Brien, R. D. (1964). *Science*, **144**, 55.
Scaife, J. F. & Campbell, D. H. (1959). *Canad. J. Biochem.*