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COMPARISON OF ASSAY METHODS FOR STUDYING *O,O*-DIETHYL, *O-p*-NITROPHENYL PHOSPHATE (PARAOXON) DETOXICATION *IN VITRO* *†‡

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Abstract—Three different techniques were used to study the inactivation of paraoxon (*O,O*-diethyl, *O-p*-nitrophenyl phosphate) by animal tissues *in vitro*. The first procedure (spectrophotometric technique) measures the amount of *p*-nitrophenol released during the hydrolysis of paraoxon (4×10^{-4} M). The second technique estimates the hydrolysis of paraoxon ($7{\cdot}7 \times 10^{-3}$ M) by manometric measurement of CO_2 liberated in a bicarbonate buffer (manometric technique). The third procedure (anti-CHE technique) measures the loss of anticholinesterase (anti-CHE) activity of paraoxon (1×10^{-7} M) after incubation with tissues. The kinetics of the reactions, tissue distributions, sex and species comparisons, the effects of calcium, magnesium and EDTA, and the effects of pretreatment *in vivo* with organophosphate esters were studied. The influence of these factors differed depending upon whether the inactivation of paraoxon was measured by the spectrophotometric or the anti-CHE technique. This indicated that the inactivation mechanism operating in the anti-CHE system was not the same as paraoxon hydrolysis as measured by the spectrophotometric and manometric techniques. The results of studies of the effects of incubation time, substrate concentration, dialysis, cofactors and pretreatment with organophosphate esters suggest that tissue binding of paraoxon is the principal mechanism of inactivation operating in the anti-CHE system

PARAOXON is a potent inhibitor of acetylcholinesterase (CHE). It is the toxic metabolite formed in animal tissue from the widely used insecticide, parathion (*O,O*-diethyl, *O-p*-nitrophenyl phosphorothioate).[1,2] Paraoxon is often present as a contaminant of technical and commercial grades of parathion and it can be formed nonenzymatically from parathion during storage at room temperature, by heating and by the action of ultraviolet light.[3]

Until recently, evidence obtained by studying the metabolism of parathion *in vitro* indicated that parathion was detoxified only after it had been converted to paraoxon.[4] Therefore considerable research has been devoted to studies of the mechanisms of detoxication of paraoxon by animal tissues.

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Aldridge[5] demonstrated that paraoxon is enzymatically hydrolyzed *in vitro* by enzymes (A-esterases) present in plasma and tissue homogenates of various laboratory animals. The hydrolysis products are not cholinesterase inhibitors and the reaction, therefore, represents a detoxication mechanism.

Since Aldridge's original work, the detoxication of paraoxon *in vitro* has been the subject of several investigations. Usually two different approaches have been used. One approach is to measure the products of enzymatic hydrolysis either by the direct spectrophotometric measurement of *p*-nitrophenol or indirectly by manometric measurement of CO_2 released in a bicarbonate buffer.[6–8] The other approach is to measure the loss of anticholinesterase activity of paraoxon after incubation with tissues;[9–11] this will be referred to as the anti-CHE technique. Relatively high concentrations (10^{-4} to 10^{-2} M) of paraoxon are used with the spectrophotometric and manometric techniques and low concentrations (10^{-8} to 10^{-6} M) for the anti-CHE, technique. The first approach has the advantage of direct measurement of the products of a specific enzyme-catalyzed reaction, while the anti-CHE method has the theoretical advantage of measuring all reactions occurring in the assay system which result in a decrease in the primary toxic action of paraoxon.

A comparison of the results obtained by several authors revealed that these two approaches gave different results. Murphy[11] used both procedures to study species differences in paraoxon inactivation by liver homogenates *in vitro*. The relative activities of livers from different species varied depending upon the method of assay. Krueger and Casida[8] used the spectrophotometric technique to compare the amount of paraoxon degraded by homogenates of normal and paraoxon-resistant houseflies. They demonstrated that similar paraoxon hydrolysis occurred with both strains, despite the fact that one strain was 6·7 times as resistant to topically applied paraoxon. On the other hand, Van Asperen and Oppenoorth,[9] working with an anti-CHE system and a substrate concentration 250,000 times less than that used by the preceding workers, obtained a good correlation between the level of resistance to paraoxon and the ability of homogenates from their strains to inactivate paraoxon.

These observations prompted a reinvestigation of methods used to study paraoxon inactivation *in vitro* in the hope that information would be obtained which would permit selection of proper methods for studying metabolism–toxicity relationships.

MATERIALS AND METHODS

Chemicals

Paraoxon and parathion were supplied by the American Cyanamid Co., Princeton, N.J. They were of the highest purity (99·8 per cent pure) available. Tri-*o*-tolyl phosphate (TOTP, technical grade) was purchased from the Eastman Organic Chemical Co., Rochester, N.Y. Nicotinamide adenine dinucleotide phosphate (NADP), glucose 6-phosphate, flavin adenine dinucleotide (FAD) and reduced glutathione (GSH) were obtained from Sigma Chemical Co., St. Louis, Mo.

Animals and preparation of tissue samples

The animals used in this study were adult male and female Holtzman rats (200–250 g) adult male Swiss–Webster mice (25–30 g), adult male guinea pigs (300–400 g) and chickens (Leghorn cockerels 300–350 g). The animals were housed in air-conditioned rooms and were supplied with food and water *ad libitum*.

The animals were sacrificed by decapitation and exsanguination. Blood was collected in heparinized centrifuge tubes and the plasma and red blood cells were separated by centrifugation at 600 *g* for 10 min. The tissues were quickly removed, blotted, weighed and homogenized at 0° in the appropriate solution with a Potter–Elvehjem all-glass homogenizer. For the spectrophotometric and anti-CHE procedures, tissues were homogenized in deionized distilled water containing 1·15% potassium chloride. For manometric measurements of paraoxon hydrolysis, homogenates were prepared in 0·026 M sodium bicarbonate buffer that had been gassed with CO_2 for 15 min and adjusted to pH 7·6. Unless otherwise stated, all tissue samples were kept at 0–4° until used and all incubations were completed within 4 hr after the animals were sacrificed.

Assay Procedures

Spectrophotometric method. Enzymatic formation of *p*-nitrophenol from paraoxon (paraoxonase activity) was measured according to a procedure similar to the one described by Neal and DuBois.[4] The assay system contained appropriate amounts of whole tissue homogenate or plasma, 400 mμmoles paraoxon, 0·2 ml of 0·2 M sodium phosphate buffer, pH 7·6, and sufficient deionized water to make a final volume of 1 ml. The substrate solution was prepared just before use by appropriate dilution with water of a stock solution (0·2 M) of paraoxon in absolute ethanol that was stored in the dark at 0–4°. The small final concentration of ethanol (0·2%, v/v) in the test system had no inhibitory effect on the enzymatic activity. Incubations were carried out in air at 37° in 25-ml open beakers for 30 min in a metabolic shaker. At the end of the incubation period, 2·5 ml of cold acetone and 0·2 ml of 0·5 M glycine–NaOH buffer, pH 9·5, were added to the beakers. The mixture was transferred to centrifuge tubes, shaken, stored at − 25° for at least 30 min, centrifuged and the absorbance of the supernatant read at 410 mμ against a substrate tissue blank that was prepared by adding tissue after acetone. The results were expressed in millimicromoles of *p*-nitrophenol released per gram of tissue (wet weight) per 30 min.

Manometric method. In some experiments hydrolysis of paraoxon was measured by manometric assays of the acid products formed. For this assay, paraoxon was suspended in 0·026 M sodium bicarbonate buffer, pH 7·6, containing 5% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), and 0·5 ml of the suspension (23 μmoles paraoxon) was added to the side-arms of Warburg flasks. The main compartments of the flasks contained tissues and sufficient bicarbonate buffer to make a final volume of 3 ml. The flasks and manometers were gassed with 5% CO_2 in nitrogen for 5 min and equilibrated for 5 min at 37°. At zero time, the paraoxon was tipped into the main compartment. Manometer readings were taken at 5 min intervals. Assays were performed in duplicate and all analyses were corrected for nonenzymatic hydrolysis of the substrate and endogenous liberation of CO_2 by tissue in the absence of substrate. The results were expressed in microliters of CO_2 released per gram of tissue (wet wt.) per 30 min.

Anti-CHE technique. The capacities of the tissues to inactivate paraoxon were also determined by measuring the anticholinesterase activity of a given quantity of the compound before and after incubation with tissues. The procedure was similar to those described previously.[9–11] Paraoxon (0·5 mμmole) was incubated in test tubes for 10 min at 37° with appropriate amounts of whole tissue homogenate or plasma

in a 5-ml total volume of 0·02 M phosphate buffer, pH 7·6. The reaction was stopped by placing the tubes in a boiling water bath for 90 sec (preliminary tests showed that boiled liver did not inactivate paraoxon). Aliquots (0·6 ml) of the incubation mixtures were assayed for anticholinesterase activity by using 50 mg of homogenized rat brain as the source of the acetylcholinesterase.[12] The inhibition produced by aliquots from tubes containing tissues was compared with that produced by aliquots from a substrate blank that was carried through the same incubation, heating and storage procedures. The results were expressed as millimicromoles of paraoxon inactivated per gram of tissue (wet weight) in 10 min.

The significance of differences between means were tested by *t*-tests or analysis of variance.[13]

RESULTS

Kinetics of paraoxon inactivation

Para-nitrophenol production, as measured by the spectrophotometric technique, and the amount of paraoxon inactivated, as measured by the anti-CHE procedure, increased with increasing quantities of rat liver and plasma (Fig. 1). Time course

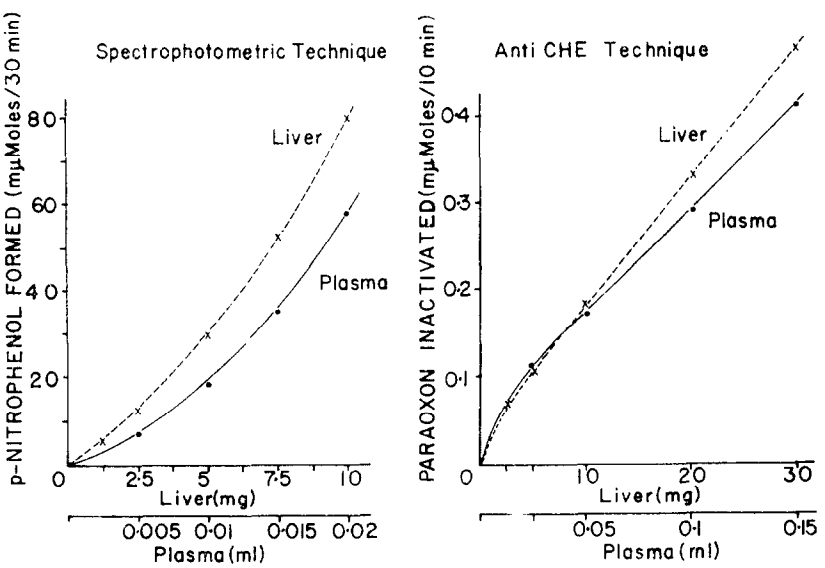

FIG. 1. Inactivation of paraxon by various quantities of adult male rat liver and plasma *in vitro*.

studies showed that while *p*-nitrophenol production increased throughout the 60 min incubation period, the disappearance of paraoxon as measured by the anti-CHE technique occurred during the first 2·5 min of incubation and then stopped (Fig. 2). A linear production of CO_2 with respect to time and tissue concentration was obtained with the manometric technique (Fig. 3). These results demonstrate that the hydrolysis of paraoxon, as measured by both the spectrophotometric and manometric techniques, followed the kinetics of an enzyme-catalyzed reaction. As seen in Fig. 4, there is the usual linear relationship between the reciprocal of the velocity of the reaction

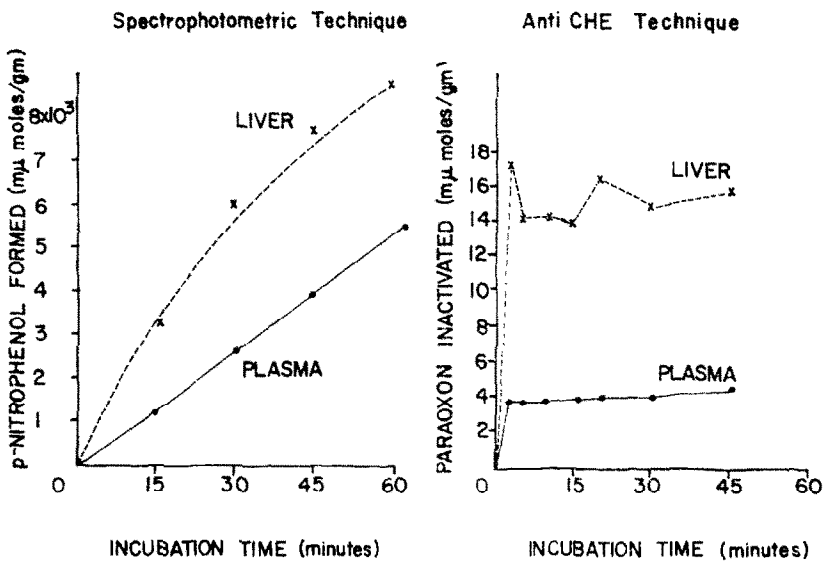

FIG. 2. Rate of paraoxon inactivation by adult male rat liver and plasma *in vitro*. The spectrophotometric assay system contained 0·1 ml of a 10% liver homogenate or 0·1 ml of a 1:5 dilution of plasma. The anti-CHE assay system contained 0·1 ml of a 10% liver homogenate or 0·05 ml plasma.

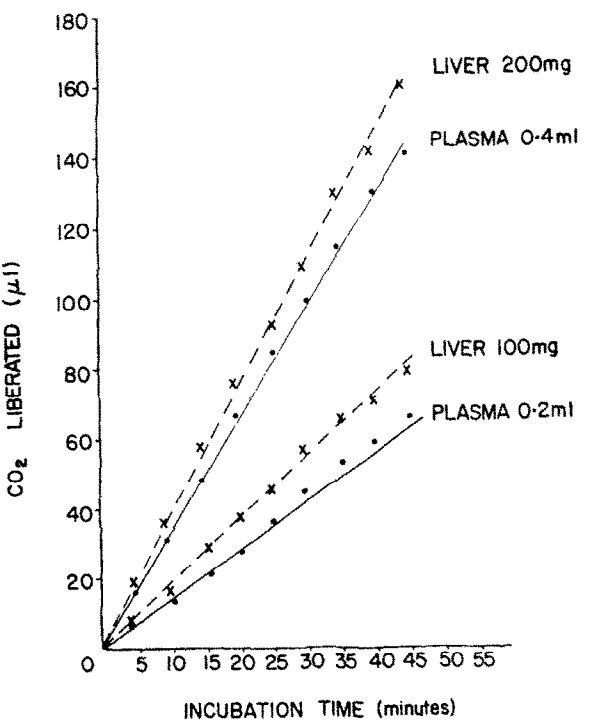

FIG. 3. Rate of paraoxon hydrolysis by adult male rat liver and plasma *in vitro* measured by the manometric technique.

(1/v) and the reciprocal of the substrate concentration (1/[S]). With the spectrophotometric technique, the maximum velocity (V_{max}) of paraoxon hydrolysis is 4 mμmoles per 5 mg liver per min and the K_m value is $8{\cdot}33 \times 10^{-4}$ M.

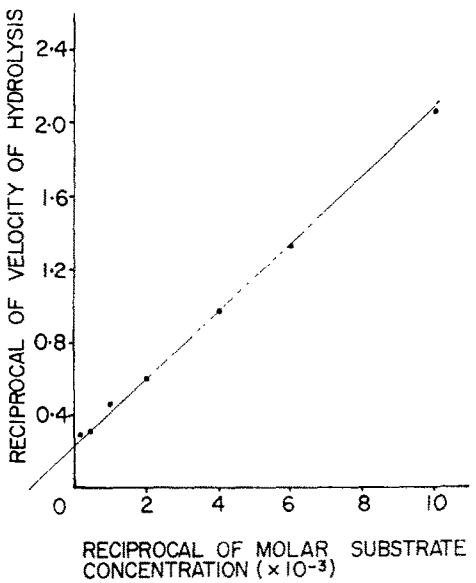

FIG. 4. Double reciprocal plot of paraoxon hydrolysis by rat liver. Five mg of homogenized male rat liver was incubated with different concentrations of paraoxon in the spectrophotometric test system described under assay procedures. The initial velocity of hydrolysis is given as mμmoles of *p*-nitrophenol liberated per min during the first 15 min of incubation.

The inactivation of paraoxon as measured by the anti-CHE technique did not exhibit the time–response relationship expected for an enzyme-catalyzed reaction. However, this type of response can occur with an enzymatic reaction when the enzyme is progressively inactivated during the incubation period or when the substrate or a cofactor is in a limited amount. Careful consideration was given to determine if these possibilities could explain the results obtained with the anti-CHE system. Experiments were performed to test whether the low substrate concentration in the anti-CHE system was a limiting factor which prevented the progressive inactivation of paraoxon with time. In one series of experiments, 0·5, 1·0 and 2·0 mμmoles paraoxon were incubated with 10 mg rat liver or 0·05 ml of rat plasma and the amount remaining in the test system was determined after 10 min of incubation. The results of four separate experiments of this type showed that the quantity of paraoxon destroyed by either liver or plasma was independent of the initial concentration of paraoxon within the range tested. In another series of six experiments a quantity of paraoxon equivalent to the quantity destroyed during the first 10 min was added to liver or plasma incubation mixtures and the incubation was continued for a second 10 min. No further inactivation occurred during the second 10 min. These results indicated, therefore, that substrate concentration was not a limiting factor for the inactivation mechanism operating in the anti-CHE system.

The possibility that paraoxon inactivation in the anti-CHE system was limited by an absence of cofactors was also considered. Nitro-reduction of paraoxon to the inactive metabolite, aminoparaoxon, *in vitro* has been reported by Hitchcock and Murphy;[14] dealkylation or oxidative cleavage of the phosphorus–aryl bond has been demonstrated *in vivo* or *in vitro* with other organophosphate esters (parathion, Ronnel, methyl parathion, Sumithion).[15–22] These alternative enzymatic detoxication mechanisms are cofactor dependent. Glucose 6-phosphate, NADP and FAD are required for nitro-reduction;[14] reduced glutathione (GSH) is required for dealkylation;[18] and glucose 6-phosphate and NADP are necessary for oxidative cleavage.[20–21] We tested the effect of addition of cofactors and of tissue dialysis on the ability of rat liver to inactivate paraoxon in the anti-CHE system. No decrease in the amount of paraoxon inactivated was observed after dialysis of rat liver homogenate against distilled water for 20 hr. Addition of NADP (0·0065 M) and glucose 6-phosphate (0·019 M), with or without FAD (0·0005 M), or addition of GSH (0·04 M) failed to enhance paraoxon inactivation. Furthermore, the absence of continuing activity with time could not be attributed to enzyme inactivation during the incubation, since we found that preincubation of the tissue for at least 15 min before addition of the substrate did not result in any loss of activity.

Effect of chemicals in vitro

Table 1 shows the effect of calcium, magnesium and sodium ethylenediaminetetraacetate (EDTA) on the inactivation of paraoxon by rat liver and plasma.

TABLE 1. EFFECT OF CHEMICALS ON THE INACTIVATION OF PARAOXON BY ADULT MALE RAT LIVER AND PLASMA *IN VITRO**

Chemicals	Per cent of control†			
	Spectrophotometric technique		Anti-CHE technique	
	Liver	Plasma	Liver	Plasma
$CaCl_2$ (1×10^{-4} M)	250 ± 7	188 ± 2	84 ± 9	84 ± 10
$MgCl_2$ (1×10^{-3} M)	53 ± 3	77 ± 7	98 ± 11	94 ± 4
EDTA (2×10^{-3} M)	16 ± 5	3 ± 1	97 ± 8	91 ± 13

* Figures are means ± S.E. of 3 experiments.

† Control values were obtained with the assay systems as described under assay procedures using 5 mg liver and 0·02 ml plasma for the spectrophotometric technique and 10 mg liver and 0·05 ml plasma for the anti-CHE technique.

As reported by Aldridge,[23] Main,[7] Erdös and Boggs,[24] and Neal,[21] calcium stimulated and EDTA and magnesium inhibited *p*-nitrophenol production by rat liver and plasma. In contrast, we found that these chemicals had no significant effect on paraoxon inactivation as measured by the anti-CHE procedure. It was also observed that Tween 20 (2%, v/v) and Triton X-100 (0·8%, v/v) reduced *p*-nitrophenol production by rat liver and plasma by 80–90 per cent. Four % ethanol reduced *p*-nitrophenol production by 50 per cent. In view of this finding it is probable that the manometric test system which contained 0·8% Triton X-100 underestimates the capacity of tissues to hydrolyze paraoxon.

Distribution in rat tissues

The capacities of various rat tissues to detoxify paraoxon were tested with the spectrophotometric and anti-CHE methods. With the spectrophotometric procedure, liver and plasma exhibited the highest activity; kidney, ileum and brain had no appreciable activity (Table 2). A different pattern of distribution was obtained with the

TABLE 2. INACTIVATION OF PARAOXON BY VARIOUS TISSUES OF ADULT MALE RAT

Tissue	Spectrophotometric technique* *p*-nitrophenol formed (mμmoles/g/30 min)		Anti-CHE technique† paraoxon inactivated (mμmoles/g/10 min)
	Without Ca^{++} added	Ca^{++} added (1×10^{-4} M)	
Liver	7200 ± 453	16,800 ± 931	15·20 ± 1·22
Plasma	2769 ± 227	4790 ± 516	3·67 ± 0·55
Kidney	37 ± 9	81 ± 13	5·05 ± 0·14
Ileum	19 ± 9	34 ± 8	1·70 ± 0·26
Brain	0	8 ± 2	0·64 ± 0·16

* The enzyme activity was measured using 5 mg liver, 0·02 ml plasma and 60 mg kidney, ileum and brain. Figures are means ± S.E. of 3 animals.
† The experiment was conducted using 10 mg liver, 0·05 ml plasma, 30 mg kidney, 50 mg ileum and 100 mg brain. Figures are means ± S.E. of 6 animals.

anti-CHE system. Liver was the most active tissue, followed by kidney, plasma, ileum and brain. Livers and plasma from 4 female rats were also tested and compared with values obtained from male rats. With the spectrophotometric technique, there was no significant sex difference ($P > 0{\cdot}10$) in the hydrolysis of paraoxon by rat liver and plasma. With the anti-CHE procedure, there was no significant sex difference in the inactivation of paraoxon by plasma ($P > 0{\cdot}50$), but male rat liver inactivated about 35 per cent more paraoxon than female rat liver ($P < 0{\cdot}05$).

Species comparison

A comparison was made of the inactivation of paraoxon by liver and plasma of several species. As shown in Table 3, the same type of distribution of plasma

TABLE 3. INACTIVATION OF PARAOXON BY LIVER AND PLASMA OF VARIOUS SPECIES

Tissue	Spectrophotometric technique* *p*-Nitrophenol formed (mμmoles/g/30 min)		Anti-CHE technique† Paraoxon inactivated (mμmoles/g/10 min)
	Without Ca^{++} added	Ca^{++} added (1×10^{-4} M)	
Liver			
Rat	(7)‡ 7609 ± 303	(4) 18,652 ± 1160	(41) 15·2 ± 0·83
Guinea pig	(6) 3765 ± 295	(4) 8760 ± 701	(6) 45·4 ± 2·31
Mouse	(4) 6289 ± 596	(4) 16,516 ± 503	(11) 13·3 ± 0·52
Chicken	(3) 61 ± 7	(3) 57 ± 3	(5) 12·8 ± 0·70
Plasma			
Rat	(6) 2880 ± 186	(4) 4810 ± 401	(37) 3·3 ± 0·15
Guinea pig	(6) 941 ± 115	(4) 1164 ± 169	(9) 0·6 ± 0·06
Chicken	(3) 16 ± 7	(3) 26 ± 2	(4) 0·2 ± 0·07

* Incubates contained 5 and 2·5 mg of rat, guinea pig and mouse liver, 40 mg of chicken liver, 0·02 and 0·01 ml of rat plasma and 0·3 ml of guinea pig and chicken plasma. Figures are means ± S.E.
† Incubates contained 10 mg of rat, mouse and chicken liver, 5 mg of guinea pig liver, 0·05 ml of rat plasma and 0·2 ml of guinea pig and chicken plasma. Figures are means ± S.E.
‡ Number of animals (all male).

activities was found with the spectrophotometric and anti-CHE techniques. The distribution of the liver activities differed depending upon the method selected. *Para*-nitrophenol production was slightly lower in mouse than in rat liver ($P = 0{\cdot}05$). Guinea pig liver produced only half as much *p*-nitrophenol from paraoxon as rat liver ($P < 0{\cdot}05$) and chicken had essentially no activity. On the other hand, with the anti-CHE technique, guinea pig liver exhibited a significantly higher activity ($P < 0{\cdot}05$) than rat liver, but rat liver did not differ significantly from mouse and chicken liver activity ($P > 0{\cdot}05$).

Effect of pretreatment in vivo *with organophosphate esters*

Lynch and Coon[25] reported that 24 hr after oral pretreatment of mice with TOTP (400 mg/kg) the intraperitoneal toxicity of parathion was increased. Their results suggested that TOTP pretreatment stimulated the rate of conversion of parathion to its active metabolite, paraoxon. Since this potentiation might also be the result of inhibition of paraoxon inactivation, experiments were performed to test whether administration of organophosphate esters to male rats would affect the inactivation of paraoxon by their livers and plasma *in vitro*. The data in Table 4 show that no

TABLE 4. EFFECT OF ADMINISTRATION *IN VIVO* OF ORGANOPHOSPHATE ESTERS ON THE INACTIVATION OF PARAOXON BY ADULT MALE RAT LIVER AND PLASMA *IN VITRO*

Pretreatment	Amount (mg/kg)	Route†	Time‡ (hr)	No. of Animals	Per cent of control*			
					Spectrophotometric technique		Anti-CHE technique	
					Liver	Plasma	Liver	Plasma
Parathion	3	i.p.	4	4	105 ± 5		15 ± 6	0
TOTP	125	i.p.	16	5	130 ± 13	90 ± 4	0	0
TOTP	125	i.p.	96	3	103 ± 2	104 ± 3	37 ± 1	72 ± 2
TOTP	250	p.o.	16	6	116 ± 10	89 ± 12	23 ± 4	39 ± 9
TOTP	500	p.o.	16	6	120 ± 11	118 ± 21	7 ± 3	17 ± 8

* Each pretreatment group has been compared with an equivalent number of corn oil-pretreated animals. Figures are means ± S.E.

† i.p. = Intraperitoneal administration; p.o. = oral administration (single doses).

‡ Time interval between pretreatment and sacrifice.

significant decrease in *p*-nitrophenol production was observed. These results confirm Aldridge's[26] earlier finding that paraoxonase belongs to the "A-esterase" group of enzymes, which are resistant to inhibition by organophosphate esters. On the other hand, it was found that pretreatment *in vivo* of adult male rats with TOTP and parathion inhibited the inactivation of paraoxon as measured by the anti-CHE technique.

DISCUSSION

The results of this investigation demonstrate that the effects of several factors *in vivo* and *in vitro* on the inactivation of paraoxon by plasma and liver homogenates *in vitro* are different depending upon whether the inactivation is estimated by measuring

the production of *p*-nitrophenol (spectrophotometric technique) or the loss of paraoxon's anticholinesterase activity (anti-CHE technique). This suggests, therefore, that the anti-CHE system does not measure the same mechanism of paraoxon inactivation as the spectrophotometric technique, i.e. enzymatic cleavage of the phosphorus–*p*-nitrophenol bond (paraoxonase activity). This conclusion is supported by applying the kinetic data for paraoxon hydrolysis by rat liver homogenate to the conditions used for the anti-CHE system. At the substrate concentration used in the anti-CHE system, the amount of paraoxon hydrolyzed should not exceed 0·0010 mμmole per 10 mg liver per min. This value is derived by applying the formula:[27] $v = (V_{max}[S])/(K_m)$ in which v is the expected initial velocity in mμmoles per 10 mg liver per min when [*S*], the substrate concentration in the anti-CHE assay at 0 time, is 10^{-7} M and V_{max} (8 mμmoles/10 mg liver/min) and K_m ($8{\cdot}33 \times 10^{-4}$ M) are the values obtained for liver paraoxonase (Fig. 4). In fact, the actual amount inactivated is 0·15 mμmole per 10 mg liver per min or 150 times more than the calculated value. It appears, therefore, that in the anti-CHE system the amount of paraoxon inactivated by paraoxonase would be less than 1 per cent of the total amount of paraoxon inactivated.

What other paraoxon detoxication mechanism does the anti-CHE technique measure? We have demonstrated that the failure of inactivation to progress with time was not due to limited availability of substrate or cofactors or inactivation of an enzyme during the incubation period. Therefore the lack of progressive inactivation of paraoxon with time can best be explained by postulating the existence of a tissue binding mechanism. This hypothesis is supported by several reports which suggest indirectly that paraoxon can be detoxified by binding onto proteins. It is known that several enzymes other than acetylcholinesterase are inhibited by certain organophosphate esters *in vivo* and *in vitro*. Among these are: aliesterases or B-esterases, pseudocholinesterases, chymotrypsin, trypsin,[23] lipoprotein lipase,[28–30] pancreatic and hepatic lipase,[31–33] liver steroid hydroxylase,[34] phosphoglucomutase,[35] brain and nerve dehydrogenase,[36] and transport ATPase.[37] Except in the case of liver steroid hydroxylases, where the inhibition seems to be competitive,[34] the available evidence indicates that paraoxon, like other organophosphate esters behaves like a substrate for these enzymes and is hydrolyzed; but one of the products, the substituted phosphoric acid, remains attached to the enzyme, thus preventing regeneration of the free and active enzyme.[38] Phosphorylation of these enzymes by paraoxon is accompanied by liberation of one molecule of *p*-nitrophenol for each enzyme molecule or active site inhibited. The normal physiological functions of aliesterases and pseudocholinesterases are still unknown. They can be completely inactivated without apparent association with any deleterious effect.[3] It seems possible that these enzymes in several tissues could represent important sites of loss for paraoxon *in vitro* and *in vivo*.

A further argument in favor of the hypothesis that tissue binding is the mechanism operating in the anti-CHE assay was provided by the results of experiments in rats that were treated with organophosphate esters *in vivo*. Pretreatment with parathion and TOTP inhibited the paraoxon inactivation in the anti-CHE system, but did not inhibit the enzymatic cleavage of paraoxon (Table 4). This finding is consistent with reports, summarized above, which indicate that organophosphate esters can be irreversibly bound to several proteins *in vivo*.

An observation by Lynch[39] might be related to the same phenomenon. In attempting to measure the rate of conversion of parathion (3×10^{-6} M) to paraoxon by mouse intestinal strips with an anti-CHE system, no activity could be detected during the first 16 min. Such a lag period was absent when intestinal strips from TOTP-pretreated animals were used. These results could be expected if the initial paraoxon formed from parathion were rapidly bound to available tissue sites. That is, accumulation of paraoxon would not be demonstrable until all binding sites had been occupied. The loss of paraoxon through tissue binding could therefore explain the lag period observed with control tissues. No lag period in paraoxon accumulation would be expected with tissues of TOTP-pretreated animals because, as shown in this investigation, TOTP pretreatment inhibits paraoxon binding (Table 4).

Tissue binding has been demonstrated as an inactivation mechanism for other organophosphate esters (DFP, Sarin, Tabun).[40–44] Ramachandran[44] suggested that protein binding might be the principal mechanism for the inactivation of DFP and that enzymatic hydrolysis might play a minor role *in vivo*. Paraoxon is highly toxic to rats (approximate intraperitoneal $LD_{50} = 1{\cdot}20$ mg/kg). It is probable that after administration of a maximum tolerated dose its concentration *in vivo* is closer to the substrate concentration used in the anti-CHE assay than to that used in the spectrophotometric technique. Therefore, tissue binding might also play a significant role in the inactivation of paraoxon *in vivo*. This hypothesis is supported by other studies[45] in which we found that pretreating rats with intraperitoneal injections of TOTP (at doses which inhibited the binding but not the enzymatic hydrolysis of paraoxon) increased their susceptibility to poisoning by paraoxon by about 2-fold.

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