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UNUSUAL SUBSTRATE SPECIFICITY IN THE OXIDATIVE DEARYLATION OF PARAOXON ANALOGS BY MOUSE HEPATIC MICROSOMAL ENZYMES*

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Abstract—In previous studies with various dialkyl paraoxons, it was shown that the methyl, ethyl and *i*-propyl analogs were dearylated by hepatic microsomes almost exclusively through an NADPH-independent A-esterase, while dearylation of *n*-propyl paraoxon was NADPH-dependent. The present investigation was initiated to study the basis for this specificity. Release of *p*-nitrophenol was measured spectrophotometrically after incubation of a series of dialkyl-substituted paraoxon analogs with rat hepatic microsomal preparations. Oxidative dearylation was shown to have the characteristics of a typical mixed-function oxygenase (MFO) reaction. Methyl, ethyl, *n*-butyl and *n*-amyl paraoxons were not readily metabolized by the MFO system, though considerable dearylation of *n*-propyl paraoxon was observed. When the two alkyl substituents were not identical, only the compounds with an *n*-propyl group were metabolized in this manner. Dearylation was also observed with analogs containing certain branched chains or modified ethyl groups (e.g. 2-chloroethyl and 2-methoxyethyl). The unusual specificity for this MFO reaction was associated with the presence of a 3-carbon length chain or its steric equivalent. The reaction mechanism is most likely different from that for the analogous MFO-catalyzed dearylation of parathion analogs, as *p*-nitrophenol production from the paraoxons is not accompanied by release of the corresponding dialkyl phosphate.

The ability to catalyze the biotransformation of a large number of xenobiotics with a wide variety of structures establishes the hepatic mixed-function oxygenase (MFO) system as one of the most important detoxifying mechanisms in mammals. Several studies have shown a relationship between substrate lipophilicity and MFO-catalyzed metabolism [1–4]. The high correlation coefficients obtained for some reactions imply that steric and electronic influences play comparatively minor roles in determining the susceptibility of a compound to MFO attack, though little specific information is available regarding the importance of these parameters aside from instances of stereoselectivity between enantiomers (for examples, see Refs. 5–7). Thus, it would not seem likely that within a homologous series of compounds neighboring members of the series would differ widely in susceptibility to metabolism by the MFO system.

Parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothionate), a widely used insecticide, is readily metabolized by this enzyme complex [8] (pathways A and B in Fig. 1), while ethyl paraoxon, its oxygen analog, primarily undergoes non-oxidative dearylation [9] (pathway D) catalyzed by A-esterases (EC 3.1.1.2) [10], and, to a lesser extent, oxidative dealkylation [11, 12] (pathway C). Oxidative dearylation of ethyl paraoxon (pathway E), in contrast with parathion, has not been observed. Nakatsugawa *et al.* [13], however, found that, while microsomal dearylation of methyl, ethyl and *i*-propyl paraoxon was not significantly stimulated by NADPH, dearylation of the *n*-propyl analog was increased considerably by this cofactor, thus raising the possibility that an MFO-catalyzed dearylation is a significant detoxication reaction for some paraoxon analogs, and that this reaction may also demonstrate unusual MFO specificity between homologs.

The present investigation was undertaken to better characterize this dearylating enzyme system by (a) determining if this is indeed an MFO-catalyzed reaction, and (b) studying in greater detail the basis for the substrate specificity of the enzyme.

MATERIALS AND METHODS

Animals

All experimental animals were adult males. Swiss–Webster mice were purchased from Bellaire Acres (Danville, Ind.), Sprague–Dawley rats from Murphy Breeding Laboratories (Plainfield, Ind.), and New Zealand white rabbits from local sources. The rodents were maintained on Wayne Lab-Blox, with weathered, dry hardwood chips as litter. Grass frogs (*Rana*

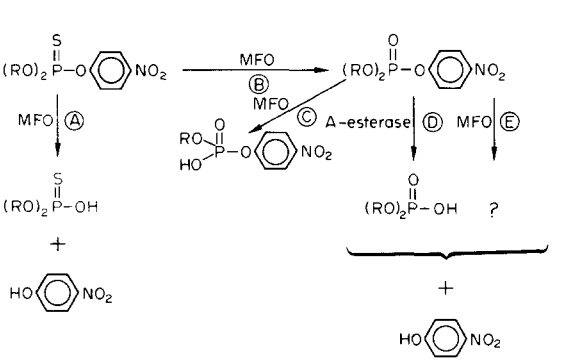

Fig. 1. Metabolic pathways for parathion analogs in hepatic microsomal fractions.

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pipiens pipiens), obtained from Mogul-Ed Corp. (Oshkosh, Wis.), were kept in a glass aquarium and were fed live American cockroaches weekly.

Chemicals

Except for glucose 6-phosphate, disodium salt (G-6-P) which was obtained from Calbiochem Co., biochemicals were purchased from Sigma Chemical Co.

Synthesis of dialkyl p-nitrophenyl phosphates

For paraoxon analogs in which the two alkyl groups are identical, the procedure of de Roos and Toet [14] was followed. For analogs in which the alkyl groups differ, the following methods were employed. *n*-Butyl ethyl and ethyl *n*-propyl phosphorochloridates were prepared according to Cadogan *et al.* [15], using pyridine as the HCl acceptor. *n*-Butyl *n*-propyl phosphorochloridate was prepared as follows: to 0.7 mole phosphorus oxychloride in 100 ml of cold ether was slowly added 0.7 mole *n*-propyl alcohol in 20 ml ether. Dry air was bubbled through the mixture to remove both the HCl and ether, and the product was carefully distilled through a variable reflux distilling head. *n*-Butyl alcohol (0.42 mole) in 20 ml ether was then slowly added to an equimolar amount of the *n*-propyl phosphorodichloridate, and the mixture was treated with dry air as before and distilled ($b_{0.15}$: 96–7°; n_D^{20}: 1.4247).

The procedure of de Roos and Toet [14] was followed for the condensation of all dialkyl phosphorochloridates with *p*-nitrophenol. Paraoxon analogs in which the alkyl groups differ were not distilled. Their purity exceeded 97 percent as determined by gas–liquid chromatography (g.l.c.).

Except where indicated, g.l.c. analyses were performed with a Tracor MT-220 chromatograph equipped with a 5-ft glass column packed with Chromosorb W (80/100 mesh) coated with 4% Apiezon N and 2% OV-225. The flame photometric detector was operated in the phosphorus mode.

Synthesis of 1,3,2-dioxaphosphorinane 2-oxides

2-Chloro-1,3,2-dioxaphosphorinane 2-oxide was prepared according to the method of Fukuto and Metcalf [16]. The *n*-propyl ester was prepared as follows: to 0.05 mole *n*-propyl alcohol and 0.05 mole pyridine in 100 ml toluene was added 0.05 mole of the chlorodioxaphosphorinane 2-oxide. After refluxing for 1 hr, the mixture was filtered and the solvent was removed by evaporation. Distillation yielded 2-*n*-propoxy-1,3,2-dioxaphosphorinane 2-oxide ($b_{0.06}$: 102–4°; n_D^{24}: 1.4430).

2-*p*-Nitrophenoxy-1,3,2-dioxaphosphorinane 2-oxide was synthesized according to Fukuto and Metcalf [16], yielding fine, white crystals (m.p. 99–100°; lit. 99–100°).

Synthesis of di-n-propyl phosphate

This compound was prepared by the method of Hoffman *et al.* [17].

Determination of hydrolysis rate constants

A 1-cm cuvette containing 3 ml of 0.01, 0.05, or 0.5 N carbonate-free sodium hydroxide was prewarmed to 37°. The organophosphate in 20 μl ethanol was added to a final concentration of 4.0×10^{-5} M, and the absorbance of *p*-nitrophenol at 400 nm was read continuously until completion of the reaction in a Unicam SP 800A recording spectrophotometer. Log $[\epsilon_\infty - \epsilon_0/\epsilon_\infty - \epsilon_t]$, where ϵ_0, ϵ_t and ϵ_∞ represent the initial absorbance, absorbance at time t, and absorbance at the completion of the reaction, respectively, was plotted against time. The line of best fit was constructed by the method of least squares, and we see from equation (1) that the slope equals $k'/2.303$, where k' is the pseudo first-order rate constant.

$$\log\left(\frac{\epsilon_\infty - \epsilon_0}{\epsilon_\infty - \epsilon_t}\right) = \frac{k't}{2.303} \tag{1}$$

Determination of ΔR_m constants

R_m values were used to determine relative lipophilicity of the paraoxon analogs. This parameter is a function of the R_f value of chromatographic systems [18], defined by equation (2):

$$R_m = \log\left(\frac{1}{R_f} - 1\right) \tag{2}$$

Ascending thin-layer chromatography was used to determine the R_f values. The stationary phase consisted of a prepared Silica gel sheet (Eastman Chromagram Sheets, No. 6060). Before use, each sheet was dipped into a solution of 5% (w/v) of Dow silicone oil 550 in hexane for 30 sec and allowed to dry. The mobile phase, consisting of acetone–water (3 : 2), was allowed to rise 16.5 cm. The compounds were visualized with 10% ethanolic KOH. The ΔR_m constants were obtained by subtracting the R_m value of methyl paraoxon from that of each of the analogs.

Preparation of microsomal pellets

For comparison of dearylating activity in different species, microsomes from untreated animals were used. Otherwise, the microsomal enzymes of mice were induced by once-daily intraperitoneal injections of sodium phenobarbital (50 mg/kg) for 4 days. The mice were starved for 24 hr after the final injections, then sacrificed. All mammals were killed by a blow on the head, whereas the frogs were pithed.

The livers were quickly excised, rinsed twice in cold 0.25 M sucrose, and homogenized in 4 vol. sucrose in a glass tissue grinder equipped with a serrated Teflon pestle. After centrifugation at 15,000 *g* for 20 min, the supernatant was spun at 176,000 *g* (average) for 40 min. The resulting microsomal pellet was washed once in 0.25 M sucrose and recentrifuged. The dry, washed pellets could be stored at −15° for at least 4 days without change in dearylating ability.

Discontinuous assay of microsomal dearylating activity

Microsomal pellets were thawed and suspended in a volume of 0.05 M Tris–HCl buffer (pH 7.6 at 37°) equal to that of the discarded supernatant above the pellet. Incubations were carried out in open 20-ml beakers. In the following order, 2.0 ml of microsomal suspension, 2.0 ml of a magnesium chloride–nicotinamide solution (each at 12.5 mM), and 1.0 ml of 6.0 mM reduced NADP were added. All reactions were initiated by the addition of 5 μl of *n*-propyl paraoxon in ethanol (0.5 mM final concentration).

The beakers were shaken at 37° and aliquots of 0.5 ml were transferred at approximately 1-min intervals into 15-ml test tubes containing 1.0 ml acetone. After centrifugation, the supernatant was withdrawn and brought to 10 ml with 0.2 M Tris–HCl buffer, pH 8.8 (25°). The absorbance of *p*-nitrophenol at 400 nm was determined with a Spectronic 20 colorimeter. The reaction was generally linear for 3–4 min, and the amount of *p*-nitrophenol produced was plotted against time.

The above procedure was employed to examine species differences and to ascertain the inducibility of the NADPH-dependent enzyme. A similar method was used to study oxygen dependency, with Thunberg cuvettes being used instead of open beakers. Into these cuvettes were placed the microsomes and magnesium chloride–nicotinamide, with NADPH and substrate in the side arm. The cuvettes were twice evacuated and refilled with nitrogen, then brought to 37°. After combining the components, the mixture was incubated for 2 min and 0.5-ml samples were withdrawn and treated as above.

Continuous assay of microsomal dearylating activity

All other determinations of either oxidative or non-oxidative dearylation were accomplished with the recording spectrophotometer. Into a 1.5-ml semi-micro cuvette were added 0.4 ml of 1.3 mM NADP, 0.2 ml of 66 mM glucose 6-phosphate (G-6-P), and 5 μl of 20 mM inhibitor when present. When carbon monoxide was added, the gas was gently bubbled through the mixture for 30 sec. The cuvette was quickly brought to 37°, at which time 2.5 units of glucose 6-phosphate dehydrogenase (G-6-P-D, EC 1.1.1.49) in 5 μl water was added. The reaction was initiated by addition of the substrate in 5 μl ethanol. The cuvettes were placed in the turbid sample position, and the absorbance at 400 nm was read continuously for 3–4 min, employing a blank containing 0.4 ml microsomes and 0.6 ml buffer. Though there was a short lag time after the initiation of the reaction, the maximum rate of *p*-nitrophenol production in each incubation was achieved within 1 min, and linearity was generally maintained for several min thereafter. NADPH-independent dearylation (A-esterase) was determined similarly, though the incubation mixture consisted of 0.4 ml microsomes, 0.6 ml buffer, and substrate in 5 μl ethanol only.

For calculation of apparent Michaelis constants and maximum velocities, a computer program modified from that of Cleland [19] was employed. Five or six concentrations of each substrate were used, and the results from replicate experiments were averaged. Protein was determined by the Biuret method [20].

Analysis of microsomal dealkylation

Into each 20-ml beaker were placed 2.0 ml of the microsomal suspension described above, 50 μl of 1.0 M nicotinamide, 2.0 ml of 1.3 mM NADP, 1.0 ml of 66 mM G-6-P, 5 μl of 0.1 M EDTA (when present), 12.5 units of G-6-P-D in 25 μl water, and substrate in 5 μl ethanol to a final concentration of 0.5 mM.

The incubations were shaken at 37° for 5 min, and 2.0-ml aliquots were transferred into 15-ml test tubes immersed in boiling water. After 90 sec the tubes were cooled and centrifuged, and unmetabolized substrate was removed from the supernatant by three extractions with 5.0-ml portions of ether. The remaining monoalkyl *p*-nitrophenyl phosphate was extracted after acidification and determined via diazomethane realkylation and g.l.c. analysis as described by Shafik and Enos [21]. Experiments with samples containing monomethyl *p*-nitrophenyl phosphate gave 85 per cent recovery by this method.

RESULTS

Physicochemical properties of the paraoxon analogs

Boiling points and refractive indices. These data are presented in Table 1. Further checks on identity and purity of these compounds were made by thin-layer chromatography on Silica gel.

Hydrolysis rate constants. The pseudo first-order rate constants (k') calculated from equation (1) were used to ascertain the susceptibility of the aryl-phosphate bond to nucleophilic attack. A few compounds (the 2-chloroethyl, methyl and allyl derivatives) were hydrolyzed so rapidly that 0.01 N NaOH was used instead of 0.05 N. The resulting rate constant was multiplied by five to obtain the corresponding value at 0.05 N. The 1-ethylpropyl analog of paraoxon was so nonreactive that 0.5 N NaOH was required, and this rate was also corrected to 0.05 N. Comparative studies of hydrolysis rates of the allyl and 2-methoxyethyl analogs by both 0.01 and 0.05 N NaOH confirmed the appropriateness of these simple corrections.

As expected from the increasing steric hindrance, k' for saturated, unsubstituted derivatives generally declines with increasing alkyl chain length and branching (Table 2), while addition of electronegative groups to the chain (2-methoxyethyl and 2-chloroethyl derivatives) has the opposite effect, as does unsaturation (allyl derivative).

ΔR_m constants. The ΔR_m values are presented in Table 2 and show a reasonable relationship between polarity and chain length, branching and substitution.

Properties of the NADPH-dependent dearylating enzyme system

Species variation. Rates of NADPH-dependent dearylation of *n*-propyl paraoxon were assessed in hepatic microsomes from four species. As seen in Table 3, dearylation was observed in all four species, with the mouse being the most rapid and the frog the least.

Effect of phenobarbital pretreatment on NADPH-dependent dearylation. To increase the activity of this dearylating system, the inducibility of the system by phenobarbital was tested. The rate of NADPH-dependent *p*-nitrophenol production from *n*-propyl paraoxon in incubations containing microsomes from untreated mice was found to be 9.4 nmoles/mg of microsomal protein/min, compared to 29.5 nmoles/mg/min with induced mice, approximately a 3-fold increase. Consequently, phenobarbital-treated mice were used in subsequent studies.

Effect of NADPH on the microsomal dearylation of some paraoxon analogs. Before deciding on the mouse as the experimental animal, it was necessary to establish that specificity of the NADPH-dependent reac-

Table 1. Boiling points and refractive indices of paraoxon analogs

$(R'O)(RO)P(O)O\langle\bigcirc\rangle NO_2$

Analog R = R'	Boiling point (°/mm Hg) Found	Literature	Ref.	n_D^{20} Found	Literature	Ref.
CH_3—	133–35°/0.10	151°/0.52	14	1.5179	1.5203	14
C_2H_5—	137–40°/0.15	163.5–65.5°/0.86	14	1.5052	1.5080	14
n—C_3H_7—	147–50°/0.08	156.5–59.0°/0.35–0.4	14	1.4986	1.5013	14
i—C_3H_7—	141–45°/0.12	149–59°/0.25–0.5	14	1.4900	1.4938	14
nn—C_4H_9—	155–56°/0.04	155–56°/0.02	14	1.4949	1.4977	14
s—C_4H_9—	*	†		1.4925	1.4959	14
i—C_4H_9—	159–61°/0.09	†		1.4921	1.4954	14
n—C_5H_{11}—	175–78°/0.06	165–76°/0.05–0.09	14	1.4896	1.4944	14
i—C_5H_{11}—	177–78°/0.08	†		1.4821	†	
$(C_2H_5)_2CH$—	‡	†		1.4896	†	
$(C_2H_5)(CH_3)CHCH_2$—	146–48°/0.07	†		1.4896	†	
$CH_3OCH_2CH_2$—§	190–91°/0.17	†		1.5042	†	
$ClCH_2CH_2$—	210–12°/0.08	†		1.5310	1.5315	22
$H_2C{=}CHCH_2$—	162–63°/0.19	†		1.4798	†	
C_2H_5— *n*—C_3H_7—	‖	†		1.5011	†	
C_2H_5— *n*—C_4H_9—	‖	†		1.4995	†	
n—C_3H_7— *n*—C_4H_9—	‖	†		1.4974	†	

* Would not distill; distillation bath: 225°; vacuum: 0.05 mm Hg.
† Information not available.
‡ Decomposes about 180°/0.05 mm Hg.
§ Analysis: found C: 42.91% H: 5.48%. $C_{12}H_{18}NO_8P$ requires C: 42.99% H: 5.41%.
‖ Distillation not attempted; 97 per cent (+) purity by g.l.c.

tion for *n*-propyl paraoxon was manifest in microsomal fractions from phenobarbital-treated mice as well as in those from the rat and rabbit as reported by others [13]. The NADPH-dependent and NADPH-independent rates of dearylation of several paraoxon analogs are presented in Table 4. EDTA (0.1 mM) was included for all NADPH-dependent reactions as a selective inhibitor for the competing, non-oxidative A-esterase (see later, Table 6). The clear preference of the NADPH-dependent enzyme for the *n*-propyl analog is apparent, though a slight amount of NADPH-dependent dearylation of both the ethyl and *i*-propyl analogs was also observed. The failure of the system to dearylate *n*-butyl paraoxon, an observation not previously reported, served to further accentuate the peculiar preference of this enzyme system for the *n*-propyl analog.

Only the methyl and ethyl analogs are active substrates for the A-esterase. *n*-Propyl paraoxon was only slightly dearylated by this enzyme, the route being insignificant by comparison with the NADPH-dependent reaction. No measurable *p*-nitrophenol was produced when either the *i*-propyl or *n*-butyl analog was employed as the substrate.

Effect of oxygen depletion on dearylation of n-*propyl paraoxon.* The dependency of this dearylating system on NADPH suggested it was an MFO-catalyzed reaction. Therefore, the amount of dearylation of this analog after oxygen depletion was tested (Table 5). Despite some variability (probably due to varying degrees

Table 2. Hydrolysis rate constants and ΔR_m values of paraoxon analogs

Analog	Average $k' \times 10^2$ (corrected to 0.05 N NaOH*)	ΔR_m*
Methyl	28.0	0.0
Ethyl	8.9	0.254
n-Propyl	5.9	0.597
i-Propyl	1.1	0.471
n-Butyl	5.7	0.876
s-Butyl	0.5	0.768
i-Butyl	5.1	0.855
n-Amyl	4.1	1.182
i-Amyl	3.8	1.056
1-Ethylpropyl	0.025	1.010
2-Methylbutyl	3.1	1.067
2-Methoxyethyl	19.2	0.067
2-Chloroethyl	70.5	0.400
Allyl	18.5	0.464
Butyl-ethyl†	6.5	0.640
Butyl-propyl†	5.6	0.823
Ethyl-propyl†	7.3	0.366
Phosphorinane‡	21.1	0.092

* Mean of duplicate experiments.
† Mixed ester ($R \neq R'$).
‡ 2-*p*-Nitrophenyl-1,3,2-dioxaphosphorinane 2-oxide.

Table 3. NADPH-dependent dearylation of *n*-propyl paraoxon (0.5 mM) in hepatic microsomes from four vertebrate species

Animal	Rate*,† −NADPH	+NADPH
Frog	ND‡	3.1 ± 0.3
Rabbit	ND	4.7 ± 0.3
Rat	ND	4.7 ± 0.3
Mouse	ND	9.4 ± 0.4

* Expressed as moles *p*-nitrophenol produced/mg of microsomal protein/min.
† Mean ± S. D. of three experiments.
‡ Not detectable; minimum detectable rate about 0.4 nmole/mg/min.

Table 4. Effect of NADPH on the microsomal dearylation of paraoxon analogs ($R = R'$)

$(R'O)(RO)P(O)O\langle\bigcirc\rangle NO_2$

Analog (0.5 mM)	Rate* Without NADPH (A-esterase)	Rate* With NADPH and A-esterase inhibitor†,‡
Methyl	4.5	ND§
Ethyl	9.5	1.0
n-Propyl	0.6	29.4
i-Propyl	ND	2.5
n-Butyl	ND	ND

* Rate is expreased as nmoles *p*-nitrophenol produced/mg of microsomal protein/min. Mean of at least two experiments.

† Inhibitor: 0.1 mM EDTA.

‡ Concentrations of NADPH-generating system components were as follows: 0.5 mM NADPH, 13.4 mM G-6-P, and 2.5 units/ml of G-6-P-D, with 10 mM nicotinamide.

§ Not detectable; minimum detectable rate about 0.4 nmole/mg/min.

of O_2 depletion), the data from the duplicate experiments clearly indicate a marked decrease (74.6 and 87.4 per cent) in dearylating activity after evacuation, showing that dearylation of *n*-propyl paraoxon is indeed an oxygen-dependent reaction. This decrease is very similar to that found with the well-known MFO substrate *p*-nitroanisole.

Effects of several enzyme inhibitors on dearylating activity. To further characterize the nature of this reaction, the effects of three known MFO inhibitors and of EDTA on the oxidative and non-oxidative (A-esterase) routes of dearylation were examined (Table 6). In evaluating the oxidative reaction, we employed *n*-propyl paraoxon as the substrate, whereas the ethyl analog in the absence of NADPH was used to measure the A-esterase-catalyzed reaction, as these two substrates are relatively specific for the respective enzymes under these conditions (see Table 4).

As is typical of MFO reactions, piperonyl butoxide, SKF-525A and carbon monoxide all inhibited dearylation of *n*-propyl paraoxon. The latter two inhibitors did not strongly affect the nonoxidative (A-esterase) pathway, and though the effect of piperonyl butoxide on this enzyme was not studied during this investigation, unpublished results obtained earlier in this laboratory indicate that it too fails to inhibit the A-esterase. In contrast, EDTA proved to be an excellent selective inhibitor of this enzyme without affecting the NADPH-dependent system. Thus, the dependence upon NADPH and oxygen, coupled with the inhibition by three known MFO inhibitors, strongly implies that dearylation of *n*-propyl paraoxon is an MFO reaction, and distinguishes it from the A-esterase pathway.

Table 5. Effect of oxygen depletion on the dearylation of *n*-propyl paraoxon and on the demethylation of *p*-nitroanisole by mouse hepatic microsomes

	Expt. No.	*p*-Nitrophenol produced* Air	N_2	% Decrease
n-Propyl paraoxon (0.5 mM)	1	54.3	13.8	74.6
	2	52.5	6.6	87.4
p-Nitroanisole (0.5 mM)	1	27.5	6.4	76.7
	2	29.0	6.1	79.0

* Expressed as nmoles/mg of microsomal protein in 2 min.

Table 6. Effects of EDTA and several MFO inhibitors on the microsomal dearylation of paraoxon analogs

Treatment	Oxidative* Rate‡	% Inhibition	A-esterase† Rate‡	% Inhibition
No inhibitor	18.2	0.0	9.0	0.0
EDTA (0.1 mM)	18.1	0.7	0.4	95.6
CO	3.2	82.4	8.6	3.7
Piperonyl butoxide (0.1 mM)	9.0	50.5	—	—
Piperonyl butoxide (0.4 mM)	3.5	80.8	—	—
SKF-525A (0.1 mM)	12.7	30.0	8.7	3.3

* Substrate for oxidative dearylation reaction was 0.2 mM *n*-propyl paraoxon.

† Substrate for A-esterase dearylation reaction was 0.5 mM ethyl paraoxon.

‡ Expressed as nmoles *p*-nitrophenol produced/mg of microsomal protein/min. Mean of duplicate experiments.

Substrate specificity of MFO dearylation of paraoxon analogs

To better circumscribe the substrate specificity of the oxidative dearylation reaction, apparent K_m and V_{max} values were determined for a series of paraoxon analogs with differing alkyl substituents (Table 7). EDTA was included in all incubations to selectively inhibit the A-esterase. The standard errors for individual K_m values generally varied between 5 and 10 per cent, while those for V_{max} were usually less than 5 per cent. The compounds employed as substrates included analogs with straight-chain alkyl groups 1 to 5 carbons in length, branched isomers of these, unsaturated alkyl groups, and carbon chains substituted with electronegative groups.

No oxidative dearylation of the methyl, *n*-butyl, *n*-amyl or *i*-amyl analogs was observed. Interestingly, however, all other compounds tested were dearylated, some of them rapidly, indicating that *n*-propyl paraoxon by no means was the only excellent substrate for the MFO-dearylating system. The K_m values were generally quite low, ranging from 0.014 to 0.058 mM. The rate of oxidative dearylation of ethyl paraoxon was slow enough (1.0 nmole/mg of protein/min at 0.5 mM substrate) to preclude determination of either K_m or V_{max} Though the rate for the *i*-propyl analog was also very slow, both constants were obtained.

To test the dependence of functions of K_m or V_{max} on lipophilic, electronic or steric influences from these substituents, multiple linear regression was employed,

Table 7. Apparent K_m and V_{max} values for oxidative dearylation of paraoxon analogs ($R = R'$)

$(R'O)(RO)P(O)O\langle\bigcirc\rangle NO_2$

Analog	$K_m \pm$ S. E.* (10^5 M)	$V_{max} \pm$ S. E.* (nmoles/mg/min)
Methyl	†	†
Ethyl	‡	‡
n-Propyl	2.7 ± 0.3	30.0 ± 1.0
i-Propyl	2.3 ± 0.2	3.6 ± 0.2
n-Butyl	†	†
s-Butyl	1.4 ± 0.1	23.7 ± 0.9
i-Butyl	5.8 ± 0.3	35.6 ± 1.0
n-Amyl	†	†
i-Amyl	†	†
l-Ethylpropyl	1.5 ± 0.2	21.9 ± 0.8
2-Methylbutyl	3.4 ± 0.3	17.8 ± 0.6
2-Methoxyethyl	4.9 ± 0.4	15.3 ± 0.7
2-Chloroethyl	1.4 ± 0.1	13.7 ± 0.1
Allyl	1.7 ± 0.2	21.7 ± 1.0

* K_m and V_{max} values are means of at least two replicates. The S. E. values for the individual K_m or V_{max} determinations were generally similar and were also averaged.

† No *p*-nitrophenol production was observed in 4 min with 0.5 mM substrate.

‡ K_m and V_{max} could not be determined due to the low reaction rate, which was 1.0 nmole/mg/min with 0.5 mM substrate.

using K_m, $\log(K_m)$, $\log(1/K_m)$, V_{max}, $\log(V_{max})$, or $\log(1/V_{max})$ as dependent variables, and k' (hydrolysis rate constants), ΔR_m, Taft's σ^* [23], and Taft's E_s [23] as independent variables. The square of the multiple correlation coefficients (R^2) for all possible combinations of independent variables with each dependent variable was calculated. No R^2 value was greater than 0.697, indicating that, singly or in any combination, the above parameters do not accurately predict these functions of K_m or V_{max}.

Closer examination of Table 7, however, produces the interesting observation that, in general, those analogs with alkyl chains of 3 carbons in length are readily dearylated, while the others are not. For example, *s*-butyl and 2-methylbutyl may be thought of as "l-methylpropyl" and "2-ethylpropyl," respectively, and both are active substrates. The shortest straight chain formed by the *n*-butyl, *i*-amyl and *n*-amyl analogs, which are not dearylated, is 4 or 5 carbons in length. A chlorine atom is approximately isosteric with a methyl group, and thus the 2-chloroethyl group is about equal to a 3-carbon chain in length. This is displayed more graphically in Fig. 2, where V_{max} for the analogs tested is plotted against the length of the alkyl chain in angstroms (Å), calculated as the distance from the center of the alkyl ester oxygen to the limit of the van der Waals radius of the terminal hydrogen atom. With the exception of 2-methoxyethyl paraoxon, all readily dearylated analogs have alkyl chains about 6 Å in length, with the length of "active chains" ranging from 5 to 7 Å. This raises the question of whether a 6 Å chain length is the major determinant of MFO dearylation of this series of compounds.

To test this hypothesis, MFO dearylation of certain analogs in which the alkyl groups are not identical was also investigated (Table 8). Compounds with all

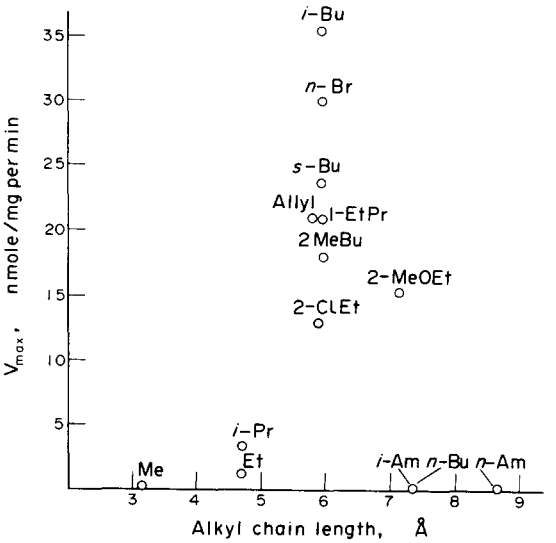


Fig. 2. Relationship of alkyl chain length to rate of oxidative dearylation of paraoxon analogs.

possible combinations of ethyl, *n*-propyl and *n*-butyl groups were studied. We found that only paraoxon analogs containing at least one 3-carbon chain are readily dearylated. It is also significant that, as with ethyl paraoxon, the butyl–ethyl analog is also slightly metabolized in this manner. Thus, it appears that only one *n*-propyl group need be in the molecule to allow rapid oxidative dearylation to occur, and that to a minor extent an ethyl but not an *n*-butyl group can substitute for the *n*-propyl.

To further characterize the substrate specificity of this reaction, 2-*p*-nitrophenyl-1,3,2-dioxaphosphorinane 2-oxide was synthesized and tested as a substrate for oxidative dearylation (Table 8). No production of *p*-nitrophenol was observed, showing that a cyclized 3-carbon chain cannot substitute for a free *n*-propyl group.

Table 8. Apparent K_m and V_{max} values for oxidative dearylation of paraoxon analogs ($R \neq R'$)

$(R'O)(RO)P(O)O\langle\bigcirc\rangle NO_2$

Analog		$K_m \pm$ S. E.* (10^5 M)	$V_{max} \pm$ S. E.* (nmoles/mg/min)
R	*R'*		
n-Propyl	Ethyl	3.7 ± 0.2	23.4 ± 0.5
n-Propyl	*n*-Propyl	2.7 ± 0.3	30.0 ± 1.0
n-Propyl	*n*-Butyl	2.8 ± 0.2	15.5 ± 0.4
Ethyl	Ethyl	†	†
Ethyl	*n*-Butyl	†	†
n-Butyl	*n*-Butyl	‡	‡
Phosphorinane§		‡	‡

* K_m and V_{max} values are means from at least two replicates. The S. E. for the individual K_m or V_{max} determinations were generally similar and were also averaged.

† K_m and V_{max} could not be determined due to the low reaction rate, which was 1.0 nmole/mg/min with 0.5 mM substrate.

‡ No *p*-nitrophenol production was observed in 4 min with 0.5 mM substrate.

§ 2-*p*-Nitrophenoxy-1,3,2-dioxaphosphorinane 2-oxide.

Table 9. Microsomal dealkylation of paraoxon analogs ($R = R'$)

$(R'O)(RO)P(O)O\langle\bigcirc\rangle NO_2$

Analog (0.5 mM)	NADPH	EDTA (0.1 mM)	% Dealkylation*
Methyl	–	–	5.4
	–	+	4.9
	+	–	7.0
	+	+	6.3
Ethyl	–	–	0.6
	–	+	0.6
	+	–	7.7
	+	+	8.8
n-Propyl	–	–	ND†
	+	+	1.6

* Per cent of initial substrate that undergoes *O*-dealkylation in 5 min.

† None detectable.

Reactions competing with oxidative dearylation

The presence of EDTA in all oxidative incubations eliminated A-esterase-catalyzed dearylation as an effective competitor for the paraoxon analogs.

Another possible competitive reaction is the microsomal oxidative *O*-dealkylating system (pathway C in Fig. 1). Dealkylation of several paraoxon analogs by microsomes in both the presence and absence of NADPH was studied in a separate experiment (Table 9). In the presence of NADPH, methyl and ethyl paraoxons are dealkylated only about 6–7 and 8–9 per cent, respectively, in 5 min, depending on whether EDTA is present or not. Even less of the *n*-propyl analog (1–2 per cent) undergoes this reaction. By contrast, approximately 30 per cent of *n*-propyl paraoxon at this same concentration (0.5 mM) is oxidatively dearylated within 4 min.

An interesting circumstance was observed regarding the routes by which methyl and ethyl paraoxons are dealkylated. Whereas ethyl paraoxon is apparently dealkylated by the MFO system, the analogous reaction involving the methyl analog appears to be largely NADPH-independent. It is unlikely that demethylation was catalyzed by soluble glutathione transferases (e.g. see Ref. 24), as washed microsomes were used.

Mechanism of oxidative dearylation

It is possible that the MFO-catalyzed dearylation of these paraoxon analogs proceeds by a mechanism similar to that for parathion analogs (pathway A in Fig. 1). For this to remain a viable possibility, di-*n*-propyl phosphate would have to be produced *in vitro*. Therefore, *n*-propyl paraoxon was incubated for 6 min as described for the discontinuous assay in Materials and Methods. Samples (4 ml) were withdrawn and methylated as described for the microsomal dealkylation experiments. G.l.c. analyses were performed with a column packed with 3% Carbowax 20M on Gas Chrom Q (80/100 mesh).

Of the 0.76 mg of organophosphate initially added to the 5-ml incubation, less than 1 per cent was converted to di-*n*-propyl phosphate, despite the fact that about 40 per cent was oxidatively dearylated, as determined by *p*-nitrophenol production. As 70–75 per cent of the di-*n*-propyl phosphate incubated and treated similarly in spiked samples could be recovered, little of this compound could have been lost by metabolism.

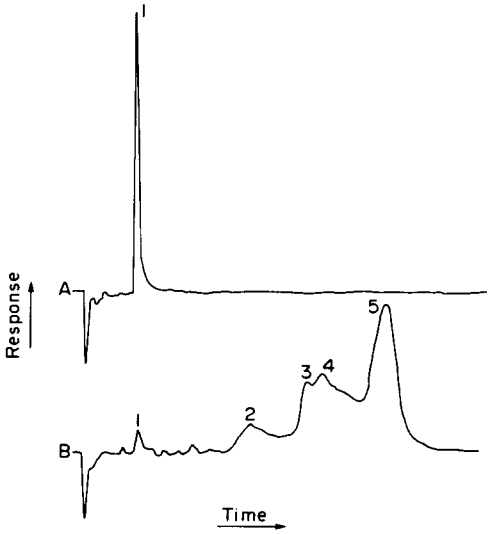
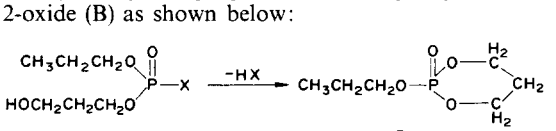


Fig. 3. Phosphorus-specific g.l.c. tracings of methylated extracts from microsomal incubations of di-*n*-propyl phosphate and *n*-propyl paraoxon. (A) Alkylated extract (1.5 μl) from incubation of 0.2 mM di-*n*-propyl phosphate with microsomes and NADPH. Peak 1 represents 55 ng of di-*n*-propyl methyl phosphate. (B) Alkylated extract (4.0 μl) from incubation of 0.5 mM *n*-propyl paraoxon with microsomes and NADPH. Peak 1 has the same retention time as, and represents about 3 ng of, di-*n*-propyl methyl phosphate. Peaks 2–5 are unknown phosphorus-containing metabolites, reproducibly obtained from such incubations.

The g.l.c. tracing resulting from the analysis of the *n*-propyl paraoxon incubation with the phosphorus specific detector was unexpectedly complex (Fig. 3). Besides the probable di-*n*-propyl methyl phosphate (peak 1), at least four other phosphorus-containing compounds (peaks 2–5) were detected in the methylated extract, none of which correspond to *n*-propyl paraoxon. Control experiments revealed that none of these compounds originated from NADPH; peaks 2–5 were observed only when microsomes, *n*-propyl paraoxon, and NADPH were present simultaneously.

In our search for likely explanations of these observations, we considered the possibility of a mechanism based on intramolecular catalysis similar to the activation reaction of tri-*o*-cresyl phosphate (TOCP). Specifically, if *n*-propyl paraoxon undergoes ω-hydroxylation, an intramolecular reaction may occur, resulting in the loss of the *p*-nitrophenoxy moiety (X) and yielding 2-*n*-propoxy-1,3,2-dioxaphosphorinane 2-oxide (B) as shown below:



If this mechanism were operating, we would expect to find compound B as a product, provided it is not further metabolized.

n-Propyl paraoxon (0.5 mM) was again incubated with microsomes and NADPH, followed by extraction and g.l.c. analysis as described in Materials and Methods. Compound B was not detected, and the greater than 85 per cent recovery of 0.02 mM compound B from spiked controls indicates it is stable under the experimental conditions.

DISCUSSION

The *n*-propyl paraoxon-dearylating enzyme located in the microsomal fraction of mouse liver is shown to be NADPH- and oxygen-dependent, with very little of the organophosphate undergoing this reaction in the absence of either. This dependency, together with the inhibition by three known MFO inhibitors and the induction by phenobarbital, indicates that *n*-propyl paraoxon dearylation has the characteristics of a typical MFO reaction.

Regarding substrate specificity of this reaction, we have shown that mouse hepatic microsomes exhibit the same specificity for paraoxon analogs, as reported previously for rats and rabbits [13]: of the paraoxon analogs initially tested in this work (methyl, ethyl, *n*-propyl, *i*-propyl and *n*-butyl), only *n*-propyl paraoxon was rapidly dearylated. The two explanations for this odd specificity that seemed most likely were as follows: (1) all of the analogs are potentially susceptible to oxidative dearylation, but other microsomal pathways rapidly metabolize most of the analogs, leaving only *n*-propyl paraoxon available for oxidative dearylation, or (2) more likely, some inherent quality of the *n*-propyl analog either directly or indirectly enables its dearylation by the MFO system, this feature being absent in other, closely related molecules.

The two known routes of metabolism of paraoxon analogs in microsomal fractions most likely to compete with the oxidative dearylating system are dealkylation and A-esterase dearylation (pathways C and D in Fig. 1). The possibility of A-esterase dearylation outcompeting oxidative dearylation for the methyl, ethyl, *i*-propyl and *n*-butyl analogs was eliminated by including EDTA as a selective inhibitor of this calcium-dependent enzyme. The striking preference for *n*-propyl paraoxon remained. Besides, the *i*-propyl and *n*-butyl analogs are poor A-esterase substrates (unpublished observations) as well as poor substrates for oxidative dearylation.

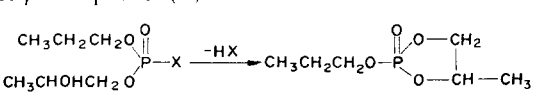
The other potential competitor, dealkylation, could not be eliminated quite as easily, as the *O*-dealkylation of ethyl paraoxon has also been shown to be an MFO reaction [11]. Consequently, dealkylation of several paraoxon analogs was determined independently. About 9 per cent of the ethyl analog underwent dealkylation in 5 min. Rates of oxidative dearylation, however, were determined within the first 4 min, during which time approximately 30 per cent dearylation occurred. Quantitative recoveries in experiments with monomethyl paraoxon indicated that this compound and probably the other mono-alkyl products of dealkylation are not further metabolized by the microsomal fraction to any great extent. Thus, dealkylation of paraoxon analogs is clearly not rapid enough to remove either methyl or ethyl paraoxon and thereby account for the marked specificity of the MFO system for *n*-propyl paraoxon. Also, the K_m for oxidative dearylation is very low, making this reaction an excellent competitor itself for potential substrates. We must conclude, therefore, that a peculiar characteristic of the *n*-propyl paraoxon molecule itself must account for its susceptibility to dearylation by the MFO system, as observed in our initial experiments.

Since only those compounds for which kinetic constants could be obtained could be analyzed, regression analysis was of limited use in understanding the relationship of physicochemical parameters to substrate specificity. Consequently, the most important variation—that between analogs that are dearylated and those that are not—could not be represented in our regression equations.

Unfortunately, the steric parameter E_s does not specificially measure what may be the most important characteristic of all, i.e. shape. It also fails to accurately account for mutual drug–enzyme perturbations. There is, however, a surprisingly good correlation between susceptibility to oxidative dearylation and a 6 Å length alkyl chain. The only exception to this generality is the rapid dearylation of 2-methoxyethyl paraoxon, which has a chain length greater than 6 Å and is more related sterically to the inactive *n*-butyl analog. With this qualification, all compounds in the series that are dearylated rapidly have an alkyl group very close to 6 Å in length. This criterion becomes even more apparent when the dearylation rates of the mixed esters are compared (Table 8).

The understanding of the reason for this 6 Å (3-carbon) chain prerequisite is the key to resolving the nature of the reaction mechanism. The strict requirement of chain length suggested the analogy with the activation of TOCP [25]. With this compound, hydroxylation at the *o*-methyl group, i.e. the third carbon atom from the ester oxygen, is followed by cyclization through an intramolecular reaction. A similar mechanism with *n*-propyl paraoxon might account for the narrow substrate specificity observed. However, none of the cyclized (phosphorinane) derivative that would result from such a reaction was found in the incubation medium. Besides, this mechanism (ω-hydroxylation) alone would not account for the activity of 2-chloroethyl and 2-methoxyethyl paraoxons.

We also considered the possibility that ($\omega - 1$)-hydroxylation of the *n*-propyl moiety might result in cyclization to a phospholane with the concurrent loss of *p*-nitrophenol (X):



A B

Unfortunately, to test this hypothesis required the synthesis of 4-methyl-2-*n*-propoxy-1,3,2-dioxaphospholane 2-oxide (compound B), and attempts to synthesize this compound failed.

It is still possible that a 6 Å chain is necessary for the molecule to assume a specific conformation that either renders the aryl-phosphate linkage suceptible to attack or allows the P=O moiety to form a peroxide-type linkage with an incoming oxygen atom, as described by Ptashne *et al.* [26] for phosphorothionates such as parathion. However, in contrast to

our results with paraoxon analogs, Wolcott *et al.* [27] found little difference in either K_m or V_{max} for the MFO dearylation of the methyl, ethyl, *n*-propyl and *n*-butyl analogs of parathion. If this mechanism were operating, the high degree of steric specificity with the phosphates compared to the relatively nonspecific dearylation of the phosphorothionates would need explanation, as would our failure to detect production of di-*n*-propyl phosphate. The complicated g.l.c. tracings, however, suggest a more complex mechanism—for example ($\omega - 1$)-hydroxylation followed by aryl-phosphate cleavage. The resulting phospholanes are relatively unstable and subject to rapid hydrolysis at both the cyclic and exocyclic groups [25]. Without knowledge of the products, further speculation on the nature of the reaction mechanism is premature.

The results of this investigation indicate that substrate specificity of the MFO-catalyzed dearylation of paraoxon analogs relies primarily on the presence of alkyl chains of specific length.

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