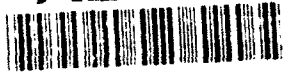


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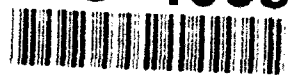
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The purpose of this project is to develop techniques that account for interspecies differences in the pharmacokinetics of xenobiotics. The hypothesis proposed is that toxicity occurs after exposure of the target organ to a characteristic concentration of toxicant for a particular period of time. To test the hypothesis, experiments were proposed to characterize the pharmacokinetics of three representative chemicals (lindane, pentachlorophenol and paraoxon) in small trout via gill exposure, and large trout and rats via intravascular injection. Compartmental toxicokinetic models were to be used. The fraction of a dose of each test compound converted to each of its metabolites by the test animals were to be determined and account for possible metabolic differences that might contribute to interspecies differences in toxicity. Binding of the substances in blood to formed elements and plasma proteins were to also be characterized. The LC50s and LD50s of the test compounds were to be determined and the values were to be converted to free concentrations using various toxicokinetic transformations. The transformation that gave a common concentration for toxicity in the three groups of animals were to be an "index of relative exposure" that would provide an estimate of the dose to the target organ rather than the dose to the animal. The area under the free concentration-time curve was to be the starting point for development of the exposure index. Successful development of such an index should result in substitution for research purposes of the index for mammalian species, and in a better understanding of interspecies differences in the dosage of chemicals that produce toxicity. The research was also to provide useful information about the toxicokinetic and toxicologic properties of the compounds.

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ABSTRACT

The purpose of this project is to develop techniques that account for interspecies differences in the pharmacokinetics of xenobiotics. The hypothesis proposed is that toxicity occurs after exposure of the target organ to a characteristic concentration of toxicant for a particular period of time. To test the hypothesis, experiments were proposed to characterize the pharmacokinetics of three representative chemicals (lindane, pentachlorophenol and paraoxon) in small trout via water exposure, and large trout and rats via intravascular injection. Compartmental toxicokinetic models were to be used. The fraction of a dose of each test compound converted to each of its metabolites by the test animals were to be determined to account for possible metabolic differences that might contribute to interspecies differences in toxicity. Binding of the test substances in blood to formed elements and plasma proteins were to also be characterized. The LC50s and LD50s of the test compounds were to be determined and the values were to be converted to free concentrations using various toxicokinetic transformations. The transformation that gave a common concentration for toxicity in the three groups of animals were to be an "index of relative exposure" that would provide an estimate of the dose to the target organ rather than the dose to the animal. The area under the free concentration-time curve was to be the starting point for development of the exposure index. Successful development of such an index should result in substitution for research purposes of fish for mammalian species, and in a better understanding of interspecies differences in the dosage of chemicals that produce toxicity. The research was also to provide useful information about the toxicokinetic and toxicologic properties of the test compounds.

INTRODUCTION

Work was focussed on paraoxon, a directly acting inhibitor of acetylcholinesterase (ACHE) and a potent toxicant of the cholinergic nervous system. This compound was chosen for study first because it has a quantifiable toxicity, namely inhibition of ACHE. Simultaneous determination of the concentration-time profile of paraoxon and its inhibition of ACHE was believed to provide a good opportunity to realize the objectives of the project. Maxwell, Vlahacos and Lenz published results of a study of another ACHE inhibitor, soman, that supports this belief (Toxicology Letters 43:175-188, 1988). They successfully modeled the soman-induced inhibition of ACHE in the rat after intramuscular injection.

Our first objective was to identify the site of action of paraoxon. While it inhibits ACHE in all tissues, the tissue in which inhibition results in death is not known for certain. The previous progress report (9-15-88 to 6-30-90; AFOSR-88-0345) described results in rat suggesting that the site of action was the CNS, but outside the blood brain barrier.

The HPLC method for determination of paraoxon that we developed proved too insensitive (limit of 1 nM or 275 ng/ml plasma) for the development of the toxicokinetic model for paraoxon and during the reporting period considerable effort was expended investigating gas chromatographic methods. Recent advances in capillary GC technology have lowered the limits of detection of compounds present in trace quantities. The GC that we used was a Hewlett Packard 5890A, series II, with electron capture, nitrogen-phosphorous and flame ionization detectors, dual capillary injection ports, autoinjector with 100 sample tray, and HP Chemstation and HP model ES/12 PC for instrument control, data acquisition and analysis.

ASSAY DEVELOPMENT PROCEDURES

A series of experiments to develop a method based on the nitrogen-phosphorous detector were carried out. This detector proved to be extremely unreliable. While paraoxon does not contain a halogen atom, we found by serendipity that the electron capture detector was very sensitive to paraoxon and this detector was much more stable than was the N-P detector. After optimization of all the instrument operating conditions, we achieved a sensitivity of 6 ng/ml for paraoxon and 5 ng/ml for parathion, a widely used organophosphate insecticide that is converted to its active form, paraoxon, in vivo. The method uses a 25 m 5% phenylmethyl silicone column, splitless injection, an automatic injector, and the following temperature program: initial temperature 70 °C, initial time 1 min; ramp at 50 °C per min to 200 °C and hold for 8 min; ramp at 70 °C per min to 250 °C and hold for 3 min. The other operating conditions are:

Injecton port temperature:	250 °C
detector temperature:	300 °C
carrier gas:	nitrogen
total flow rate:	50 ml/min.
septum purge:	0.7ml/min
column head pressure:	12 psi
total run time:	15.3 min.

We used this analytical method to develop an extraction method for simultaneous extraction of paraoxon and parathion from plasma and tissue. All sample residues were dissolved in 0.1 to 1.0 ml of iso-octane and 1-ml aliquots were injected. Malathion was used as an internal standard. The retention times were: paraoxon = 9.6 min; malathion = 10.5 min; parathion = 11.3 min.

Plasma was extracted three times with 3 ml hexane, using methanol and sodium chloride as protein denaturing agents. The extracts were centrifuged for 3 min at 5 °C and 3000 x g. The supernate was collected and evaporated under a stream of nitrogen. The residue was reconstituted in isooctane, which was subject to capillary GLC. Standard curves for parathion and paraoxon were generated in plasma; the extraction efficiency was > 93 %. The lower limit of detection was 5 ng/ml for parathion and 6 ng/ml for paraoxon.

Assay precision. Intraday variation (% relative standard deviation) was < 3.9%, and interday variation < 4.3%.

Sample stability. The stability of paraoxon and parathion in stored samples of plasma was characterized as follows: rainbow trout plasma was spiked with paraoxon and parathion separately and stored for various times at -20 °C. The plasma samples were assayed for paraoxon and parathion; the results indicated that paraoxon and parathion were stable in the frozen condition:

Stability of Paraoxon and Parathion in Plasma at -20 °C.

Time (day)	0	1	2	3	4	7	23
Paraoxon ($\mu\text{g/ml}$)	0.188	0.174	0.187	0.186	0.187	0.176	0.174
Parathion ($\mu\text{g/ml}$)	0.186	0.182	0.176	0.185	0.185	0.186	0.182

With the above analytical methodology, toxicokinetic and toxicodynamic studies of parathion and paraoxon were carried out in rainbow trout and male Fischer 344 rats.

RAINBOW TROUT

Toxicokinetics

Experimental Design. Rainbow trout (330-550 g; Fresh Water Farms, Urbana, Ohio) were acclimated to 12 °C for at least 4 weeks in 500 liter fiberglass aquaria. The trout was anesthetized with tricaine (100 mg/liter) and fitted with a dorsal aortic cannula in the dorsal buccal cavity, which was exteriorized through the anterior, dorsal region of the head. After a 24 hr recovery period, paraoxon (5.7 mg/kg) in 100 μl of 50:50 propylene glycol and water or parathion (11 mg/kg) in 50 μl DMSO containing 5 % water was administered as a rapid injection into the blood. Immediately after injection the cannula was rinsed three times with blood, by pulling blood into the cannula and pushing back into the fish to remove paraoxon or parathion bound to the cannula tubing and prevent contamination of the initial blood samples.

Serial blood samples (0.1 to 0.4 ml; 5ml for last sample) were removed at various time intervals (paraoxon, 5, 10, 15, 30, 45, 60, 90, 120, 180, and 300 min; parathion, 0.167, 0.333, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 24 and up to 500 hr.). The total volume of blood samples collected was limited to less than 15% of the estimated blood volume of trout. The blood was collected in a heparinized test tube, and centrifuged. Plasma and red blood cells were separated and stored at -20 °C until analyzed by gas chromatography.

Plasma protein binding studies. The plasma protein binding of paraoxon was determined by ultrafiltration, and parathion was determined by equilibrium dialysis.

Results

Figures 1 and 2 show the plasma concentration-time profiles after an intravascular dose of paraoxon (5.7 mg/kg) and parathion (8.0 mg/kg), respectively. Pharmacokinetic parameter values were determined using both a two compartment model (NONLIN 84, for paraoxon) and a "model independent" method (residence time).

Paraoxon toxicokinetic parameters in rainbow trout

		Compartmental	Moment*	Mean
V_{ss}	ml/kg	1000	1000 ± 384	1000
CL_b	ml/hr/kg	2850	2920 ± 265	2885
$T_{1/2}$	hr	0.550	0.52 ± 0.11	0.535
f_{free}	%			52.5

* $n = 3 \pm SD$

Parathion toxicokinetic parameters in rainbow trout

V_{ss}	ml/kg	1050 ± 56		
CL_b	ml/hr/kg	13.0 ± 3.7		
$T_{1/2}$	hr	56.7 ± 16.7		
f_{free}	%	1.23 ± 0.078		

$n = 3 \pm SD$

The two substances have very similar apparent volumes of distribution, even though the binding to plasma proteins is remarkably different. Parathion has a larger log P value than does paraoxon (3.76 vs. 2.5) and this higher fat solubility would indicate that parathion would tend to concentrate more in fatty tissue and perhaps in other tissues, leading to a larger apparent volume of distribution. However, this tendency was apparently offset by the much higher binding of parathion in the plasma. The free fraction for paraoxon in trout plasma is 175 times that for parathion. This difference in binding appears to account for most of the difference in the total body clearance values, since their ratio is 222. The much longer plasma half life of parathion also is attributable mostly to the much smaller plasma free fraction for this substance. The unbound clearance for both compounds (CL_b/f_{free}) is about $5000 \text{ ml}\cdot\text{hr}^{-1}\cdot\text{kg}^{-1}$, which is about three times the cardiac output in rainbow trout. This would suggest that exchange of these compounds across the gill epithelium is rapid (assuming that much of the clearance is due to branchial elimination) and controlled by cardiac output.

Paraoxon and parathion are poorly metabolized in trout, but have large intrinsic metabolic clearance in rat (1). Therefore, total body clearance values of parathion are expected to differ because of differences in metabolic clearance.

The observed similarity in total body clearance of paraoxon in rat and trout might be due to the plasma free fraction, which combined with the moderate lipophilicity allows significant branchial excretion in trout, a pathway not available in rat. The total body

clearance of paraoxon in trout (2.89 liter/hr/kg) was similar to the cardiac output (1.8 liter/hr/kg) suggesting significant branchial excretion in trout.

Compared with the rat, the reduced sensitivity of trout to paraoxon appears unrelated to species differences in pharmacokinetics.

Uptake kinetics of parathion. Rainbow trout (330 - 500 g; Fresh Water Farms, Urbana, Ohio) were acclimated as described in the previous section. The trout were anesthetized with tricaine (100 mg/liter) and fitted with a dorsal aortic cannula in the dorsal buccal cavity, which was exteriorized through the anterior, dorsal region of the head. After a 24 hr recovery period, trout were placed into a solution of parathion. Samples of blood were removed at various times up to 124 hours and the concentrations of parathion and paraoxon in plasma were determined along with their water concentrations.

Results

Figure 3 shows the results from this study. From these data, it appears that parathion is rapidly taken up from water. The plasma/water concentration ratio is on the order of 100 to 200, which reflects the high degree of plasma protein binding of parathion. (It should be noted that the sampling site for plasma is immediately down stream from the gill absorption site.) These data suggest that the permeability of the gill epithelium to parathion is very high and that the rate limiting factor in uptake of parathion is its removal from the absorption site by plasma. The carrying capacity of plasma is high due to the binding. Concentrations of paraoxon, formed metabolically from parathion, were also observed in some samples; its concentration was at the limit of assay sensitivity. The plasma/exposure water ratio for paraoxon is much smaller than for parathion, reflecting the lower degree of plasma protein binding of paraoxon

Toxicodynamics

Acetylcholinesterase Activity Inhibition of Paraoxon in Rainbow Trout after Water Exposure. The mechanism of acute neurotoxicity of organophosphorus insecticides is usually attributed to inhibition of acetylcholinesterase (AChE) in the central and/or peripheral nervous systems. Many of the organophosphorus insecticides, such as parathion, are phosphorothionates, which are weak anticholinesterase, and they must be metabolically activated by cytochrome P450 to the reactive oxon metabolites (i.e. paraoxon) to display toxicity. It was found that parathion can also be converted to paraoxon non-enzymatically in water, and fish could thereby be directly exposed to paraoxon.

Although a number of studies have explored the inhibition of AChE activity by paraoxon in fish, the mechanism by which paraoxon (OPs) toxicity occurs remains uncertain. The relationship of paraoxon tissue distribution and inhibition of AChE activity is considered to provide helpful information for exploring the mechanism of OPs toxicity in fish. The purpose of this study is to explore paraoxon water uptake, tissue distribution and inhibition of AChE activity in rainbow trout.

Paraoxon concentrations in tissues were determined by the assay method described above, after the tissue was homogenized (Omni Mixer Homogenizer). Brain and heart homogenate were extracted with 3 ml hexane three times using sodium

chloride and methanol as protein denaturing agents. The extraction efficiencies for brain and heart were greater than 90 % with coefficient of variation less than 10 %.

Water exposure. Trout were exposed to paraoxon by placing them into a 70 $\mu\text{g/liter}$ solution of paraoxon in a 20 gallon aquarium maintained at 12 $^{\circ}\text{C}$. The exposure times were 12, 24, 48, 72, 96, and 120 hr. The water concentration was determined before and after each exposure. At the end of each exposure time, trout were removed from the exposure solution, rinsed and anesthetized with tricane in ice water. Blood samples were removed via the dorsal aorta. After blood was withdrawn, the trout was sacrificed by a blow to the head. The tissues were collected and frozen in liquid nitrogen.

AChE activity determination. Tissues were patted dry, cut into small pieces and homogenized (Omni Mixer Homogenizer) in homogenization buffer. AChE activity was determined by Ellman assay. The Ellman assay is a colorimetric determination of AChE activity performed at the physiological temperature of the species studied (12 $^{\circ}\text{C}$ for trout). The AChE activities in tissues from paraoxon exposed trout were compared to the activity in tissues from trout which were placed in the same conditions without paraoxon present. Determination of protein in tissue homogenates was done to standardize the expression of enzyme activity in tissues; a modification of the Lowry protein assay was used.

RESULTS and DISCUSSION

Paraoxon was rapidly taken up from water, and the plasma concentration rapidly reached equilibrium with the water concentration. Changes in plasma concentration appeared to reflect the changes in brain concentration, Figure 4. Plasma AChE activity was almost completely inhibited within 48 hr; this might have resulted from the freely accessible and lower amount of AChE in plasma. Inhibition of brain AChE activity rapidly increased from 4.4% at 12 hr to 32.23% at 24 hr, then slowly increased to 42.16% at 48 hr, and reached steady state at 72 to 120 hr, Figure 5. The *in vivo* inhibition of AChE activity appears to reflect the *in vitro* slow kinetics of AChE inactivation by paraoxon in trout; the bimolecular inhibition rate constant (K_i) is about 1600 fold less in trout than in rat (2).

FISCHER 344 RATS

Pharmacokinetics of Parathion and Paraoxon after an Intravenous dose

Chemicals. Parathion and methyl parathion were obtained from Chem Service (West Chester, PA) while paraoxon was purchased from Sigma Chemical Company (St. Louis, MO). Parathion and methyl parathion were used as obtained (>98.5% purity) while paraoxon which was 95% pure, was purified by equilibrating paraoxon in a mixture of methylene chloride and 5% sodium bicarbonate. The organic phase was separated and the solvent was evaporated to leave behind paraoxon. Reagent grade solvents were used in sample analysis.

Animals and Treatments. Male, Fisher 344 rats were housed in facilities with an ambient temperature of $21 \pm 2^{\circ}\text{C}$ and a 12 hour light:dark cycle. A jugular vein cannula was implanted under ether and/or pentobarbital anesthesia and the cannula externalized

at the back. The animals were allowed to recover from surgery for two days and the cannula patency was maintained by daily infusion of heparinized saline. The animals were fasted overnight before the toxicokinetic study.

Dosing. Due to the limited solubility of both parathion and paraoxon, the dosing solution was made of 95% DMSO in water. The dosing volume never exceeded 50 μ l for all animals. The dose administered was determined accurately by analysis on the GC. The dose was administered by drawing blood into the cannula, switching to the syringe containing the dose and injecting it. The residual dose in the cannula was flushed by infusing heparinized saline. Atropine sulfate (25 mg/kg) was dissolved in isotonic saline and was administered 30 minutes prior to dosing with parathion and paraoxon.

Sampling Times. Due to the low quantities of paraoxon that were found after paraoxon dosing, blood samples were taken at 5, 10, and 15 minutes; the animals were then sacrificed individually and blood was collected at time points of 30, 45 and 60 minutes. The blood samples after parathion dosing were collected at 5, 10, 15, 25, 40, 60 min, 2, 4, 6, 8, 12, 18, and 24 hr, respectively. At each sampling point 100 μ l of blood was collected.

Protein Binding. Paraoxon was incubated with pooled rat plasma at 37° C for 90 min. To separate bound from unbound paraoxon, ultracentrifugation of the plasma was performed at 37° C for 20 min at 1,500 x g using a micropartition system (Centrifree, Amicon corporation, MA). The concentrations of paraoxon in the plasma (total concentration) and filtrate (unbound concentration) were determined by GC. Parathion protein binding was determined by equilibrium dialysis at 37° C for 24 h in a Spectrum Teflon cell dialyzer system. The buffer and plasma compartments were sampled to obtain unbound and total concentrations.

Sample Preparation and Analysis. The plasma samples were spiked with the internal standard methyl parathion dissolved in methanol (<20 ml), vortexed for 15 s and extracted thrice with 2 ml of isooctane. The combined extracts were evaporated under a stream of nitrogen and finally reconstituted in an appropriate volume of isooctane for injection onto a GC capillary column. A gas chromatograph equipped with an electron capture detector was utilized for the analysis of parathion and paraoxon. Nitrogen was the carrier gas with a flow rate of 50 ml/min and the column used was a 25m long; the liquid phase was 55% phenyl methyl silicone. The retention times of the analytes were as follows: methyl parathion, 9.3 min; paraoxon, 9.8 min; parathion, 11.4 min. The standard curves were linear in the range of 8-200 ng. The mean recovery from plasma for paraoxon was 88% with a coefficient of variation of 7.7% and for parathion it was 92% with a CV of 6.4%.

Plasma concentration-time profiles were analyzed using both model dependent and model independent methods. For model dependent analysis, the NONLIN84 toxicokinetic data analysis program was used for computer fitting. A monoexponential equation was fitted for paraoxon and a biexponential equation gave the best fit to parathion plasma concentration-time data.

Results and Discussion. Figure 6 shows the plasma concentration-time profile of paraoxon in rats given a iv bolus dose of 0.4 mg/kg. The decline of plasma concentrations was monoexponential. Paraoxon had an extremely short half-life of 0.23 hrs. The clearance was 13.2 lit/hr/kg with a volume of distribution of 4.4 lit/kg. The clearance and volume of distribution values obtained by both model dependent and

model independent methods were very close. Protein binding studies indicated that 54.5 % of paraoxon was unbound in the plasma. Figure 7 shows the plasma concentration-time profile of parathion after an iv bolus dose of 3.0 mg/kg. Parathion shows a biphasic decay with a terminal half-life of 4.13 h. The clearance was 3.09 lit/hr/kg and the steady state volume of distribution was 10.8 lit/kg. Protein binding studies indicate that parathion was extensively bound to the plasma proteins and only 2.5% was found to be unbound. The parameters obtained with both model independent and model dependent methods were in close agreement.

Compared with the 3.9 lit/hr/kg blood flow to the liver, paraoxon's clearance value of 13 lit/hr/kg was much higher, suggesting that paraoxon was cleared by non-hepatic pathways. In vitro stability studies in plasma showed that paraoxon was highly unstable and rapidly decomposed (results not shown here), and it could be that blood was at least one additional site of paraoxon clearance. On the other hand parathion's clearance was less than the liver blood flow, suggesting that parathion was being cleared mainly by the liver.

Comparison of the toxicokinetics of parathion and paraoxon in the rat shows that parathion has a half-life about eighteen times larger. Parathion's volume of distribution was about two and half times larger and the clearance about three times smaller than were paraoxon's. The large volume of distribution of parathion indicated extensive distribution into the tissues and could possibly explain the longer half-life. As parathion was eliminated redistribution from the tissues into blood takes place; since parathion was a lipophilic drug, this redistribution process could continue for a long time, resulting in a long terminal half life. The smaller clearance could be due to the smaller fraction unbound of parathion as compared with paraoxon. Parathion could be a low extraction compound and for low extraction compounds, clearance depends on the fraction unbound in blood. Since parathion was only 1.5% unbound in plasma its clearance was low as compared with paraoxon, which has 54.5% fraction unbound.

Toxicokinetic parameters of paraoxon in Fischer 344 rat

Parameters	Model dependent	model independent
$t_{1/2}$ (hr)	0.23	
kel (hr ⁻¹)	3.01	
cl (lit/hr/kg)	13.244	13.36
Vd (lit/kg)	4.4	4.442
Protein binding (%unbound)	54.5	

Toxicokinetic parameters of parathion in Fischer 344 rat

Parameter		Model dependent	Model independent
$t_{1/2\beta}$	(hr)	4.13	
C _i	(lit/hr/kg)	3.085	2.97
Vd	(lit/kg)	9.803	10.785
Protein binding (%unbound)	2.5		

Tissue distribution, Disposition and AChE activity after IV dose of Parathion

Methods. The animals were dosed with 1 mg/kg of [¹⁴C] parathion through the jugular vein and three animals each were sacrificed at 0.33, 1.16, 1.66, 3.91, 9.16 and 12.75 hrs respectively. Blood, brain, diaphragm, liver, lungs, kidneys, muscle and heart were collected, frozen in liquid nitrogen and stored at -40°C. At the last time point serial blood samples were collected at 0.83, 0.16, 0.25, 0.416, 0.66, 1, 2, 4, 6, 8, 10, 12 hrs respectively. Urine was collected at intervals of 0-4.3, 4.3-8.33 and 8.3-12.6 hrs respectively. Feces was also collected from 0-12.6 hrs. Tissues were analyzed for parathion and its metabolites after extraction, separation on HPLC, collection and counting the fractions on a Beckman LS 60001C scintillation counter. The tissues were also analyzed for the AChE activity.

Analysis. Parathion (ps), paraoxon (po), *p*-nitrophenol (pnp), *p*-nitrophenol sulfate (pnps) and *p*-nitrophenol glucuronide (pnpg) were detected by HPLC analysis. The HPLC system consisted of a dual pump solvent delivery system (Beckman pump model 126), a manual injector (Rheodyne) and a wavelength detector (Beckman 167); the sample loop held 100 µl. The column used was a reverse phase C₁₈ Econosphere, 5 micron particle size (Alltech/Applied science). A gradient system was used to separate the compounds with a 10 mM phosphate buffer, pH 2.75, (pH adjusted with phosphoric acid) and acetonitrile as the two solvent systems. The gradient started with 91% phosphate buffer and 9% acetonitrile held for 5 min, then changed to 40% phosphate buffer over a period of 10 min. After being held for 5 min at this composition, it was again changed to original composition in 3 min. The wavelength used was 280 nm and the flow rate was 1 ml/min. The retention times were as follows: *p*-nitrophenyl β-D glucuronide, 8.33 min; *p*-nitrophenyl sulfate 10.62 min; *p*-nitrophenol, 12.9 min; paraoxon, 15.23 min and parathion, 19.62 min.

Sample workup. Plasma samples were spiked with the compounds dissolved in methanol (<20 µl). The plasma was then precipitated with the addition of 200 µl of methanol, centrifuged at 9,000 x g for 2 min and supernate collected. Extraction was repeated with another 200 µl of methanol and the combined extracts were evaporated under nitrogen and injected onto HPLC after reconstituting in the mobile phase. The extraction efficiencies achieved were parathion, 60% ; paraoxon, 65%; *p*-nitrophenol, 52%; *p*-nitrophenol sulfate, 70% and *p*-nitrophenol glucuronide, 54%. **Tissues** were homogenized in 100 mM phosphate buffer pH 7.4 containing 3 mM EDTA, spiked with the compounds and precipitated with 1 ml of acetone. The homogenate was spun at 5,000 x g for 10 min, supernate collected and reduced in volume. Saturating amounts of

sodium chloride were added and extracted twice with ethyl acetate and the combined extracts were evaporated and prepared for injection onto HPLC. For liver and kidney, where all the five compounds were quantified, acidified ethyl acetate was used in place of ethyl acetate. The extraction efficiencies were as follows: parathion, 60%; paraoxon, 57%; *p*-nitrophenol, 50%; *p*-nitrophenol sulfate, 55% and *p*-nitrophenyl glucuronide, 70%. **Urine** was diluted in mobile phase and injected onto the HPLC. **Feces** were allowed to air dry and combusted in a Hewlett Packard sample oxidizer after mixing with cellulose powder. The resultant radiolabeled CO_2 collected and counted on a scintillation counter. **AChE activity** was determined as described earlier.

Results.

Profiles of parathion and its metabolites in plasma. Following the administration of the iv dose of 1 mg/kg [^{14}C]parathion, parathion, paraoxon, *p*-nitrophenol, *p*-nitrophenol sulfate, and *p*-nitrophenol glucuronide were detected in the plasma (Fig. 8). Parathion concentrations declined rapidly initially indicating a rapid distribution into the tissues and also due to elimination as can be seen from the high metabolite levels at this time and slowly later indicating a long terminal half-life as redistribution from the tissues into blood takes place. Parathion was present in higher concentrations than its metabolites at all time points. All four metabolites had peak concentrations at the first time point of 5 mins and the concentrations declined in parallel to that of parathion. *p*-Nitrophenol sulfate was present in higher concentrations among the four metabolites with paraoxon, *p*-nitrophenol and *p*-nitrophenol glucuronide levels being considerably lower than parathion and *p*-nitrophenol sulfate at all time points. Paraoxon, *p*-nitrophenol and *p*-nitrophenol glucuronide levels declined below detection limits past 12 hrs.

Tissue distribution profile. Parathion distributed very rapidly into all the tissues studied and levels reached peak concentrations by the first time point, 0.33 hrs (Figs. 9-15). Levels remained high in tissues, and except in kidneys and liver, was the compound present in highest concentrations. Levels in diaphragm, muscle, liver and lungs were higher followed by heart, kidneys, and brain. In all the tissues, parathion concentrations were lower than those in plasma and declined in parallel with plasma concentrations. Paraoxon and *p*-nitrophenol were also detected in all the tissues but the levels were low compared to those of parathion. In particular *p*-nitrophenol concentrations were higher in kidneys, liver and lungs than any other tissues. Heart and diaphragm had the lowest *p*-nitrophenol concentrations. *p*-nitrophenol could be quantified in kidneys, liver and lungs even at the last time point of 12.75 hrs whereas in brain, diaphragm and heart, it was not found after 3.91 hrs.

Paraoxon on the other hand was present in higher levels in liver followed by lungs, brain, and skeletal muscle. Lowest concentrations of paraoxon were found in diaphragm. In heart and diaphragm, paraoxon could not be quantified after 3.91 hrs whereas in brain, lungs, liver and muscle it declined below detection limits after 9.16 hrs. Looking at the tissues specifically, the concentrations of all compounds peaked at the first time point of 0.33 hrs in all the tissues. In kidneys, *p*-nitrophenol sulfate was present in highest quantities at all time points followed by parathion, *p*-Nitrophenol, *p*-nitrophenol glucuronide and paraoxon. In the case of liver too, *p*-nitrophenol sulfate was present in higher concentrations followed by parathion, but paraoxon and *p*-nitrophenol concentrations were similar and higher than *p*-nitrophenol glucuronide concentrations unlike in kidneys. In lungs, *p*-nitrophenol concentrations were higher than those of paraoxon whereas in brain, skeletal muscle and heart paraoxon

concentrations were higher. Levels of both *p*-nitrophenol and paraoxon were similar in diaphragm.

Urinary excretion profiles. The percent of dose excreted in urine as the parent compound and different metabolites at various time intervals is shown in table 3. In all the three time intervals studied *p*-nitrophenol sulfate formed the major metabolite to be excreted, comprising 30.27, 12.27, and 3.65% of the dose respectively. *p*-nitrophenol glucuronide comprised 1.88, 0.87, and 1.1% respectively in the three intervals. *p*-nitrophenol was the third metabolite excreted in the urine to the extent of 0.35, 0.14, and 0.14% respectively. No parathion or paraoxon was detected in the urine. Totally, 50.66% of the dose was excreted in the urine in the form of *p*-nitrophenol, *p*-nitrophenol glucuronide and *p*-nitrophenol sulfate in 12.6 hrs. A separate study indicated that about 85% of the dose was excreted in the urine at the end of 22 hrs.

Excretion of total radioactivity in feces. Combustion of feces collected up to 12.6 hr in this study and upto 22 hr in a separate study indicated that less than 1% of the total dose was excreted in feces.

Percent of dose excreted in the feces and in urine at various time intervals (hrs).

a) urine	0-4.5	4.5-8.33	8.33-12.6
pnp	0.35	0.14	0.14
pnps	30.27	12.27	3.65
pnpg	1.88	0.868	1.1
ps, po	nd	nd	nd
b) feces	0-12.6		
unfractionated	< 1%		

nd, not detected

AChE inhibition. All the tissues studied showed AChE inhibition after the iv dose of 1 mg/kg parathion (Figs.16-17). In particular, plasma, brain, and liver AChE was inhibited rapidly and to the highest extent than other tissues. Brain and liver AChE was 20% of the control within 2 hrs while that of plasma was 30%. By the end of 12.75 hrs Plasma, brain, and liver AChE was 20, 18, and 5% respectively. Kidneys, heart, lungs, muscle and diaphragm showed varying degrees of inhibition. Diaphragm showed the least degree of inhibition and its AChE activity was 75% of the control. Heart and kidneys AChE was 52% and 40% respectively at the end of 12.75 hrs. Skeletal muscle was inhibited relatively slowly compared with other tissues and its activity was 40% of the control at the last time point. Lungs were inhibited to 70% of the control within 2 hrs and to 60% at the end of 12.75 hrs.

Discussion. Parathion was proposed to be biotransformed by cytochrome P-450 dependent monoxygenases to paraoxon and *p*-nitrophenol and/or to diethylphosphoric acid or diethylphosphothioric acid and *p*-nitrophenol. Paraoxon can either bind to serine esterases such as AChE to cause toxicity or carboxylesterases to be inactivated or hydrolyzed by A-esterases present in plasma, liver and lungs etc. to *p*-nitrophenol and diethyl phosphoric acid. The *p*-nitrophenol produced from all these pathways is then believed to be conjugated and excreted as sulfate and glucuronide. After the iv bolus dose of 1 mg/kg [¹⁴C] parathion, it was widely distributed in different tissues and very

high levels were seen. Parathion levels persisted in all the tissues and were seen to decline in parallel with that of plasma. This was consistent with its high partition coefficient and also its very high volume of distribution seen in toxicokinetic studies. Paraoxon and *p*-nitrophenol were seen in all the tissues studied although the levels were much smaller than those of parathion and in addition *p*-nitrophenol sulfate and *p*-nitrophenol glucuronide were also detected in liver and kidney. *p*-Nitrophenol sulfate was the metabolite present in highest concentrations in both liver and kidneys with *p*-nitrophenol glucuronide present in low concentrations indicating that the *p*-nitrophenol formed undergoes predominantly sulfation rather than glucuronidation. In situ perfusion of rat livers yielded the same pattern of biotransformation of parathion (3). Parathion, paraoxon, *p*-nitrophenol, *p*-nitrophenol sulfate and *p*-nitrophenol glucuronide were recovered in the effluent from rat livers perfused under steady state conditions. Urinary excretion studies revealed that *p*-nitrophenol sulfate was the major metabolite excreted in the urine with *p*-nitrophenol glucuronide and *p*-nitrophenol forming minor metabolites. This was consistent with the observation that in general at low doses phenols are conjugated to sulfate to a greater extent than to glucuronide in rats but was in contrary to a report which characterized *p*-nitrophenyl glucuronide as the major metabolite as opposed to *p*-nitrophenol sulfate in pigs (4). Overall, the pattern of all the metabolites distribution in different tissues suggests that biotransformation of parathion was the slowest step and both paraoxon and *p*-nitrophenol as soon as they are formed are further metabolized, paraoxon to *p*-nitrophenol and *p*-nitrophenol to the sulfate and glucuronide. This was also evident from the plasma concentration-time profiles of parathion and its metabolites (Fig. 8). All the four metabolite levels are seen to decline more or less in parallel to parathion and are also much lower. From the toxicokinetic studies earlier, the half-life of paraoxon was determined to be 0.166 hrs, since *p*-nitrophenol sulfate and *p*-nitrophenol glucuronide are conjugates and are eliminated relatively faster, their formation was slower than their elimination and hence they seem to decline slowly implicating a longer half-life.

The differences, both in the rate and extent of AChE inhibition seen in the different tissues could be due to the differences such as level of paraoxon concentrations, AChE levels, carboxyl esterase levels (CaE), paraoxonase activity and possibly extra hepatic parathion metabolism contributing to the local pool of paraoxon. AChE levels are high in brain and muscle followed by liver, diaphragm, heart, lungs and kidney (5). Paraoxonase was an A-esterase that hydrolyzes paraoxon and high activities are present in liver and plasma and to a smaller extent in the lung (6). Similarly CaE which contributes to the disposition of paraoxon is present in varying quantities in different tissues. Highest activity was found in the liver, plasma and muscle followed by kidneys and lungs (5). Brain, diaphragm and heart have low activities. Although no attempt has been made here to correlate AChE activity with either paraoxon or parathion levels, the dramatic variation in the AChE inhibition in the different tissues was presumably a result of the various competing pathways that paraoxon can take and a simple correlation between paraoxon levels and AChE inhibition in the tissues was not seen. A physiologically based toxicokinetic and pharmacodynamic model incorporating all these parameters was expected to provide such a correlation and is being currently developed.

References

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Fig 3. Concentration-time profiles of parathion and paraoxon in water and plasma in Rainbow Trout exposed to water.

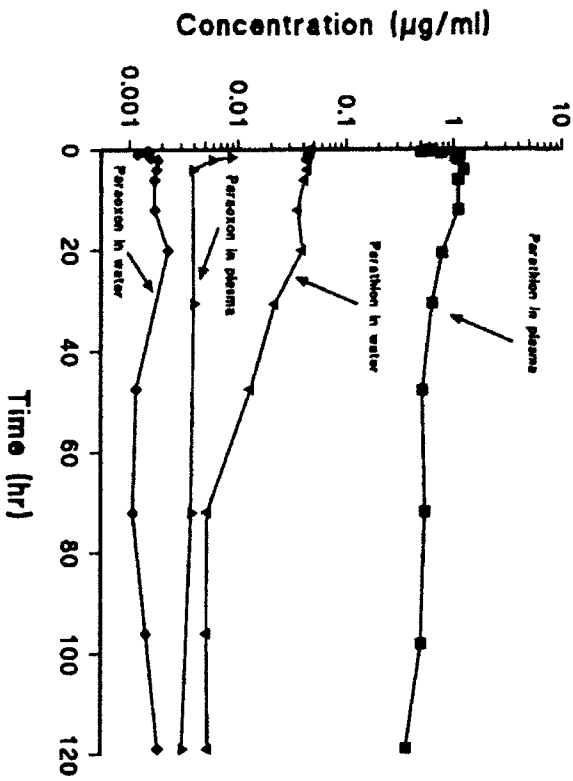


Fig 1. Plasma concentration-time profile of paraoxon after 1A dose (5.7 mg/kg) in Rainbow Trout.

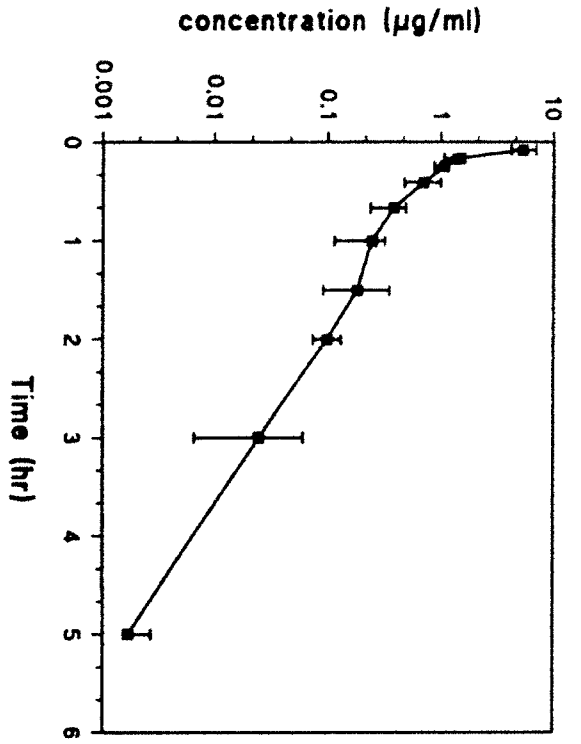


Fig 4. Concentration-time profiles of paraoxon in Rainbow Trout after water exposure.

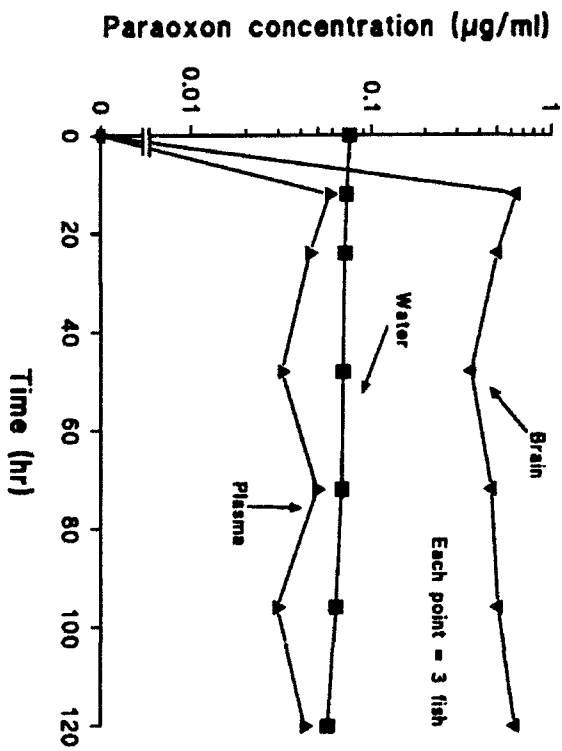
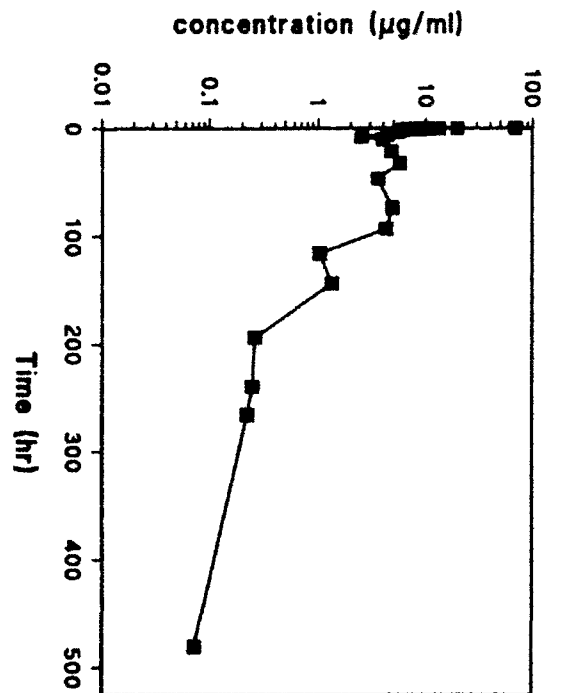


Fig 2. Plasma concentration-time profile of parathion after 1A dose (8 mg/kg) in Rainbow Trout.



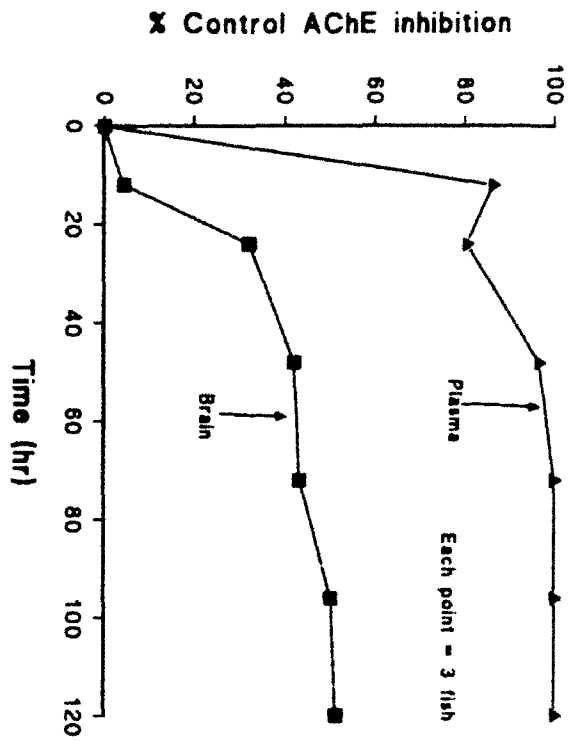


Fig 5. Time course of AChE inhibition in Rainbow Trout after water exposure.

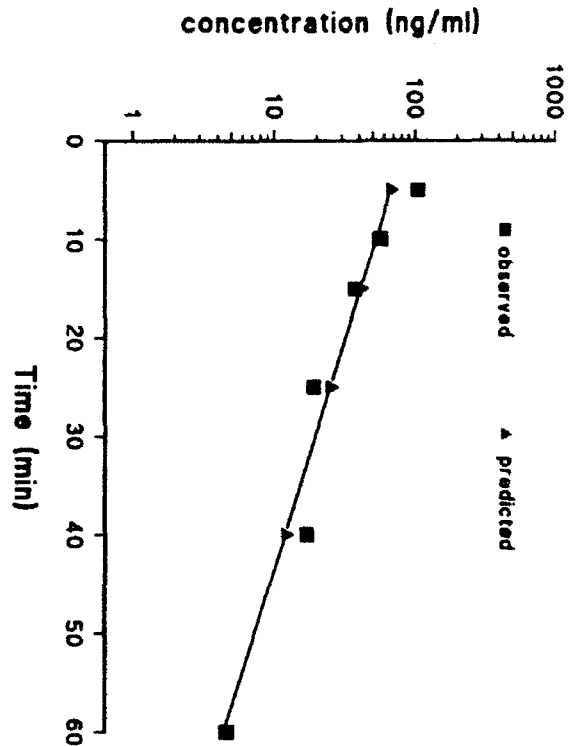


Fig 6. Plasma concentration-time profile of paraoxon after an iv dose (0.4 mg/kg)

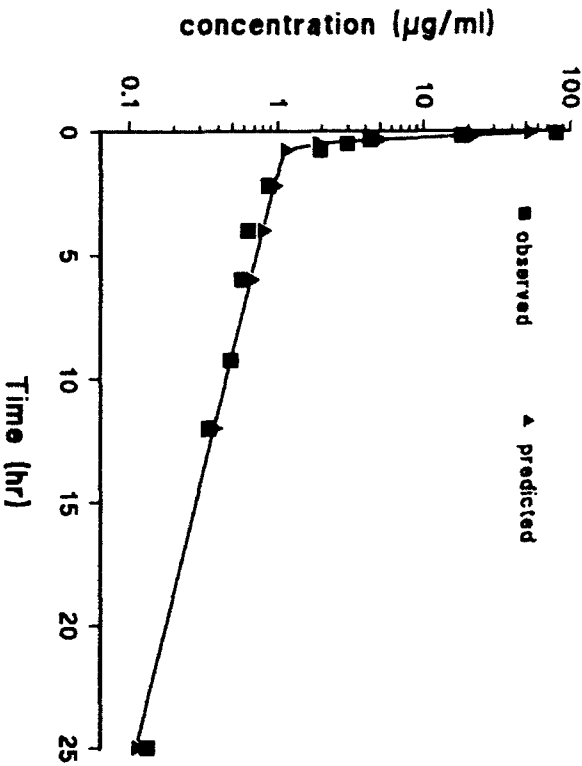


Fig 7. Plasma concentration-time profile of parathion after an iv dose (3 mg/kg)

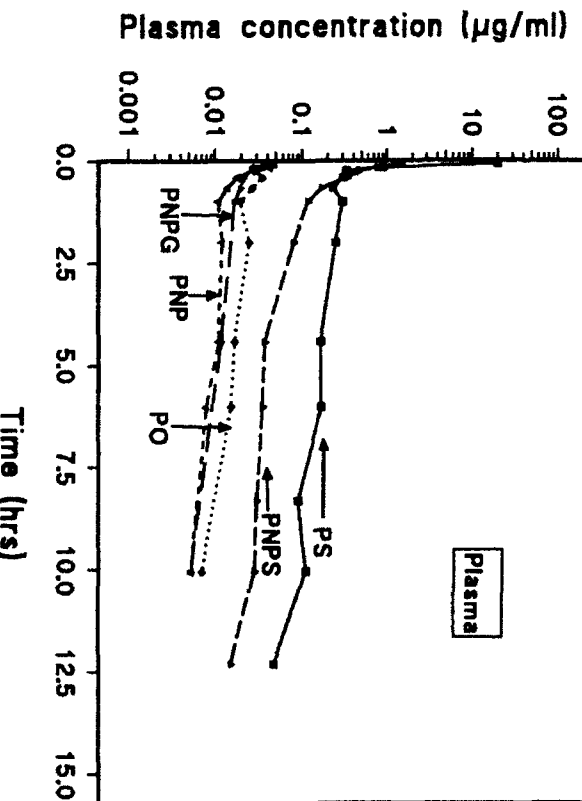


Fig 8. Plasma concentration-time profiles of parathion, paraoxon, p-nitrophenol, p-nitrophenol sulfate and p-nitrophenol glucuronide.

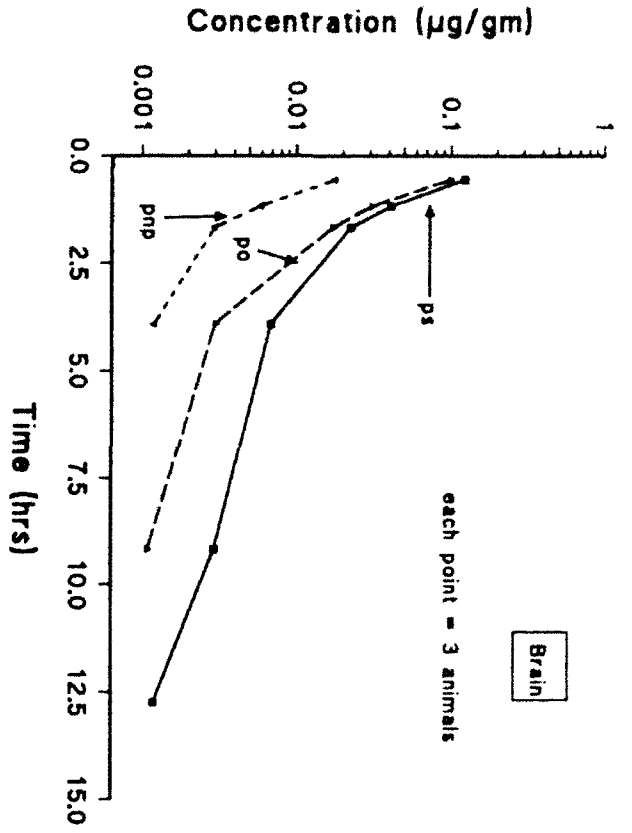


Fig 9. Concentration-time profiles of parathion, paraoxon and p-nitrophenol in Brain.

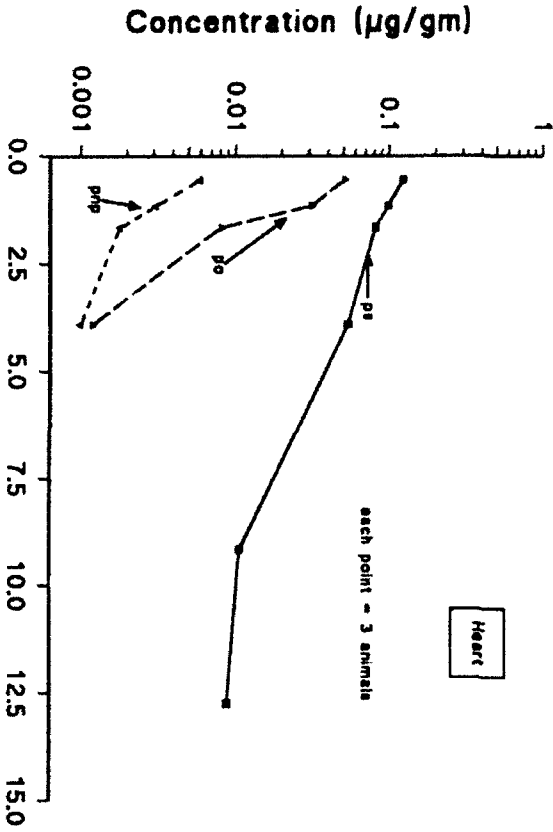


Fig 11. Concentration-time profiles of parathion, paraoxon and p-nitrophenol in the Heart.

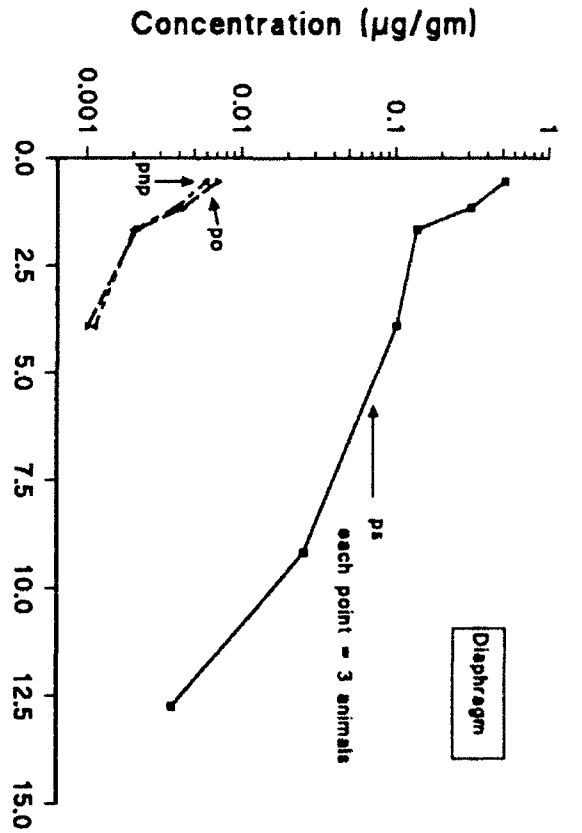


Fig 10. Concentration-time profiles of parathion, paraoxon and p-nitrophenol in the Diaphragm.

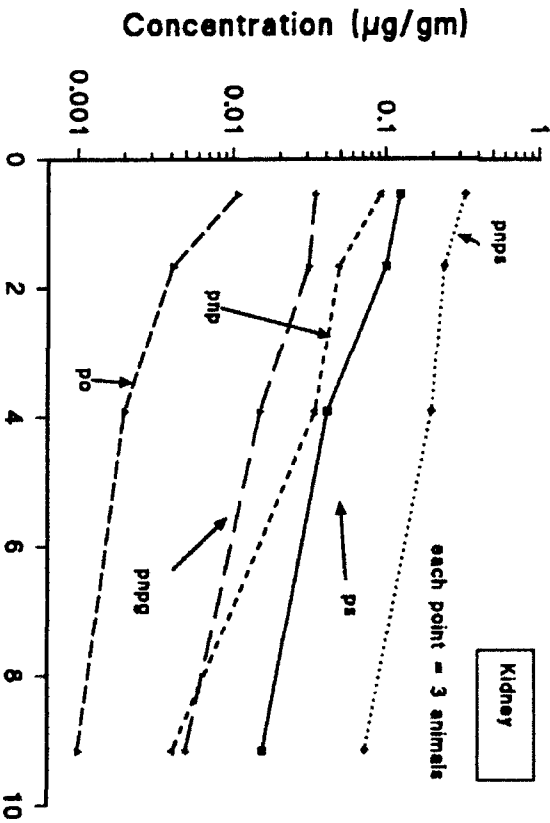


Fig 12. Concentration-time profile of parathion, paraoxon, p-nitrophenol, p-nitrophenol sulfate and p-nitrophenol glucuronide in the Kidneys.

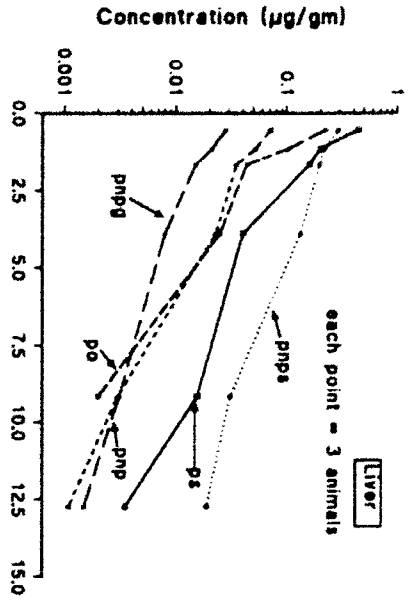


Fig 13. Concentration-time profiles of parathion, paraoxon, p-nitrophenol, p-nitrophenol sulfate and p-nitrophenol glucuronide in the Liver.

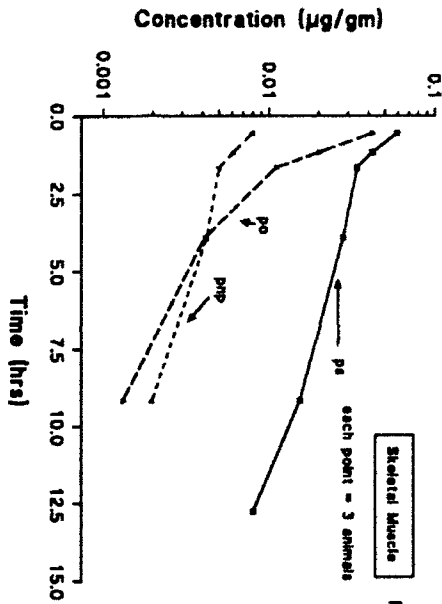


Fig 14. Concentration-time profile of parathion, paraoxon and pnp in lungs.

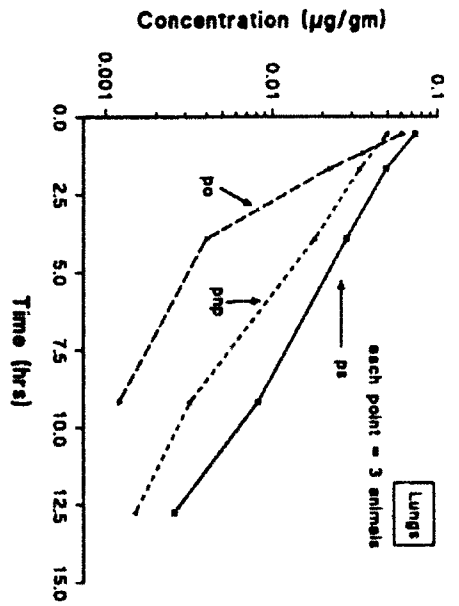


Fig 15. Concentration-time profiles of parathion, paraoxon, p-nitrophenol in the Muscle

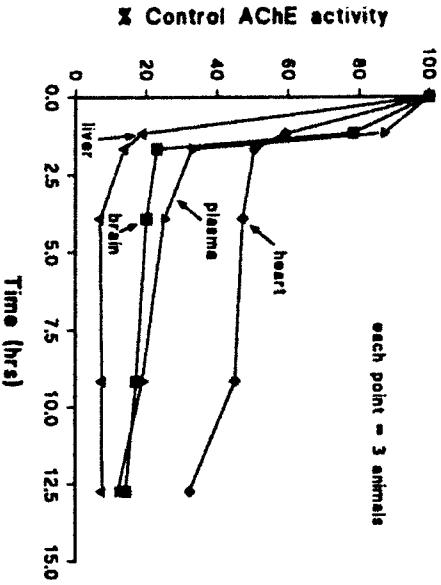


Fig 16. Time course of AChE inhibition in brain, heart, liver and plasma.

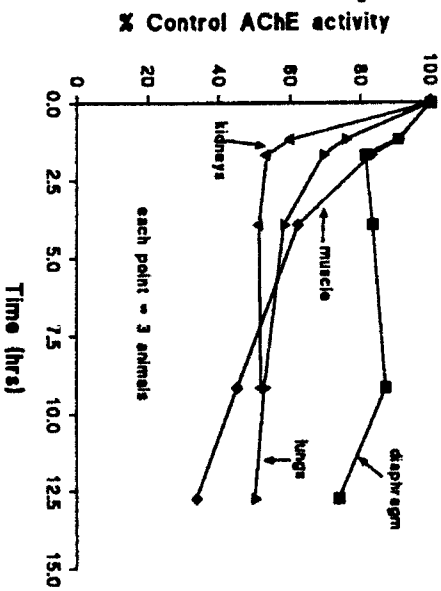


Fig 17. Time course of AChE inhibition in diaphragm, kidneys, lungs and muscle.