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13. ABSTRACT (Maximum 200 words) Work was focused on paraoxon, a direct inhibitor of acetylcholinesterase (AChE) and a potent toxicant on the cholinergic nervous system. While paraoxon inhibits AChE in all tissues, the tissue in which inhibition results in death is not known for certain. It is clear that death after acute paraoxon poisoning results from asphyxiation. The dose of paraoxon at cessation of breath (CoB) average 5.7 mg/kg at all infusion rates, which suggests that the same site of action and mechanism for paraoxon-induced CoB was in effect at all infusion rates. While heart AChE activity at CoB was independent of the infusion rate, heart appeared not to be the sensitive site since it was pumping blood at CoB. A site of action consistent with the data was CNS outside the blood-brain barrier. With low infusion rate, most of the total brain AChE was inhibited. With increasing infusion rate, inhibition of total brain AChE activity would decrease, due to less time for paraoxon to penetrate the BBB; the extra-BBB site would always be rapidly inhibited. Heart and diaphragm AChE was at the level observed at CoB while inhibition of brain AChE increased with increasing dose, again indicating brain as the sensitive site.

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Period: September 15, 1988 to June 30, 1990

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"XENOBIOTIC KINETICS AND TOXICITY AMONG FISH AND MAMMALS"

SUBMITTED TO:

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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 are 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 pharmacokinetic models will be used. The fraction of a dose of each test compound converted to each of its metabolites by the test animals will 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 will also be characterized. The LC50s and LD50s of the test compounds will be determined and the values will be converted to free concentrations using various pharmacokinetic transformations. The transformation that gives a common concentration for toxicity in the three groups of animals will be an "index of relative exposure" that will 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 will 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 will also provide useful information about the pharmacokinetic and toxicologic properties of the test compounds.

PROGRESS

During the project period work was focussed on paraoxon. This is a directly acting inhibitor of acetylcholinesterase (AChE) and is 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 have recently 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. It is clear that death after acute paraoxon poisoning results from asphyxiation. However, this could result from depression of the respiration center in the CNS, from paralysis of musculature required for breathing or from cardiac arrest. For soman, the site of AChE inhibition that causes death is apparently the brain, since AChE inhibition occurs there very rapidly, and faster than in diaphragm or muscle.

The approach to identification of the site of action of paraoxon was to administer paraoxon at a constant rate intravenously into the anesthetized test species until death occurred and then to rapidly remove tissues for analysis of AChE activity in several tissues. This experiment was repeated for several different rates of infusion of paraoxon. The site of action is the tissue that shows the same AChE inhibition at death, independent of the rate of infusion of paraoxon. This approach to identification of site of action was pioneered by G. Levy, and it has been used extensively for several drugs since its first use (M. Danhoff and G. Levy. Kinetics of drug action in disease states. I. Effect of infusion rate on phenobarbital concentrations in serum, brain and cerebrospinal fluid of normal rats at onset of righting reflex. J Pharmacol Exp Ther 229:44, 1984).

METHODS

The initial period of this project was devoted primarily to development of methods. The goals of this project require direct quantification of paraoxon and of AChE activity in tissues.

Preparation of paraoxon. Basic concerns in the use of paraoxon were first explored. Due to the chemical instability of paraoxon, frequent purification of stock solutions was necessary. The major product of paraoxon degradation is *p*-nitrophenol, which is highly visible in alkaline conditions. Using methylene

chloride and dilute sodium carbonate, *p*-nitrophenol is removed from paraoxon until the yellow color of *p*-nitrophenol is no longer visible in the aqueous phase. The amount of paraoxon present is measured after drying the organic phase and new stock solutions of the desired concentrations are prepared.

The chemical instability of paraoxon was the basis for all decontamination procedures. All contaminated glassware is held overnight in a dilute alkaline solution, which hydrolyzes the paraoxon into non-toxic products. Glassware can then be cleaned by normal laboratory procedures.

Animal tissue collection and preparation. Procedures for handling of animals and the collection and processing of tissues were developed. Rats are anesthetized with pentobarbital (IP) and cannulated at the jugular vein. Paraoxon, (in 1% ethanol in saline), is infused until a toxic end point occurs. This end point was defined as cessation of breathing (CoB) in the rat. When breathing stops, the heart continues to beat for some time (5-10 min). While it might be argued that death has not occurred at the time breathing ceases, death functionally does occur at that point. Once CoB is reached, brain damage occurs very soon thereafter and recovery does not occur. For each rat infused with paraoxon, another rat (control) was infused with 1% ethanol in saline over a similar period of time. Immediately at the end of each infusion, rats were decapitated and the brain, heart and diaphragm of each animal were collected in cold buffer. Tissue homogenates were prepared and assayed for AChE activity and protein concentration.

Some cannulation of anesthetized fish has been done. Many experimental modifications must be made to accommodate the unique characteristics of fish.

To quantify paraoxon by HPLC, a method for extracting paraoxon from tissue homogenates was developed. The paraoxon extraction procedure involved precipitation of protein from tissue homogenates with cold acetone. After centrifugation, homogenate supernatants were extracted with ethyl acetate, then dried under a stream of nitrogen. Preliminary work has shown the importance of beginning paraoxon extraction procedures as soon as possible after tissue is homogenized to ensure minimal loss of paraoxon due to its metabolism in the tissue. Preliminary work was done by adding paraoxon standards in known quantities to tissue homogenates. Extraction begun at 30 min after paraoxon addition resulted in recovery of approximately 90% of the paraoxon, as determined by HPLC.

Quantification of paraoxon by HPLC. After acquisition of HPLC hardware and Beckman's System Gold software, a procedure for quantifying paraoxon was developed. Quantification of paraoxon by HPLC involved isocratic delivery of an acetonitrile and water mobile phase over a reversed-phase silica column with UV detection. The limit of detection by this method is 1 nM, with reliable quantification possible with 2 nM or more of paraoxon. This method has some advantages over other methods reported in the literature. Paraoxon retention time is greatly reduced, allowing analysis of an increased number of samples in a given period of time. Also, the mobile phase used is relatively safe to handle as opposed to the use of methylene chloride mobile phase of other methods. In addition, the mobile phase is easy to formulate and does not require the addition of corrosive buffer salts or expensive ion-pairing agents, which are commonly used in HPLC quantification of paraoxon. In addition to quantifying paraoxon from tissue, this method was also used to verify the concentration of paraoxon in solutions administered to animals for experimental purposes.

The amounts of paraoxon found in rat tissues after paraoxon infusion are extremely small. To increase the sensitivity of paraoxon quantification by HPLC, we are developing an assay to quantify *p*-nitrophenol. This assay is based on the fact that *p*-nitrophenol is intensely colored and is therefore detectable in smaller quantities than is paraoxon. Paraoxon extracted from tissues is hydrolyzed with sodium hydroxide and one unit of *p*-nitrophenol is produced from each unit of paraoxon present in the extract. The quantity of *p*-nitrophenol is therefore equivalent to the quantity of paraoxon hydrolyzed. The HPLC assay of *p*-nitrophenol involves the use of an alkaline mobile phase over a pH-stable reversed-phase column, with a visible light detector.

Acetylcholinesterase activity. Acetylcholinesterase (AChE) activity was determined by the Ellman assay. The Ellman assay is a colorimetric determination of AChE activity performed at the physiological temperature of the species studied. The assay is quite sensitive and is the standard test used to measure this activity. Paraoxon is an inhibitor of AChE activity. As such, the Ellman assay is useful in the assessment of the effects paraoxon has on the function of vital tissues. The degree of inhibition due to paraoxon exposure varies between different species. Preliminary experiments were done to modify the Ellman assay for accurate measurement of AChE activity specifically in the rat. Enzyme activity in tissues from paraoxon-infused rats was compared to activity in tissues from vehicle-infused (control) rats to

determine which vital tissues were most affected by exposure to paraoxon. Cessation of continuous paraoxon activity in tissues isolated was accomplished by excising tissues into cold buffer containing acetylcholine. This particular technique was derived from and verified by preliminary in-vitro assays.

Determination of protein in tissue homogenates was done to standardize the expression of enzyme activity in tissues. A modification of the Lowry protein assay gave the most reliable protein values under conditions used in our laboratory. The procedure begins with a DOC-TCA protein precipitation step, which provides for recovery of proteins from interfering substances present in the buffer. The assay step is similar to the standard Lowry procedure, with absorbance measured at 660 nm. BSA is utilized to determine the standard curve each time the assay was used.

RESULTS

The following results have been obtained:

TABLE 1. Results of paraoxon infusion experiment in the rat; each value is the mean of three \pm SE.

Infusion Rate (mg/hr)	Lethal Dose (mg)	Time to Death (hr)	% AChE Inhibition		
			Brain	Heart	Diaphragm
Bolus	1.87 \pm 0.20	-. ^a	27.7 \pm 3.7	90.5 \pm 3.7	90.2 \pm 4.0
4.0	1.77 \pm 0.37	0.44	23.8 \pm 14	87.0 \pm 12	78.9 \pm 13
3.26	1.73 \pm 0.07	0.53	28.0 \pm 3.3	82.8 \pm 0.8	73.3 \pm 1.6
2.47	1.60 \pm 0.17	0.65	42.1 \pm 6.6	79.3 \pm 3.6	62.2 \pm 0.9
1.6	1.53 \pm 0.07	1.04	76.3 \pm 1.7	81.4 \pm 4.8	47.0 \pm 12.9
0.8	1.74 \pm 0.11	2.2	94.2 \pm 2.1	80.8 \pm 3.5	2.1 \pm 1.1

^aToo short to measure.

The following control AChE activities were observed; they agree with published values:

TABLE 2. Acetylcholinesterase and protein concentrations (mean \pm SE) in brain, heart and diaphragm in control rats.

	n	Brain	Heart	Diaphragm
AChE nmol \cdot min ⁻¹ mg protein ⁻¹	8	48.3 \pm 5.3	18.6 \pm 3.1	6.03 \pm 0.8
Protein mg g tissue ⁻¹	8	32.3 \pm 2.5	68.2 \pm 14.8	45.7 \pm 13.6

DISCUSSION

The esterase inhibition versus infusion rate data are also depicted in Figure 1. The dose of paraoxon at CoB averaged 5.7 mg/kg at all infusion rates, which suggests that the same site of action and mechanism for paraoxon-induced CoB was in effect at all infusion rates. While heart AChE activity at CoB was independent of the infusion rate, heart appeared not to be the sensitive site since it was pumping blood at CoB.

A site of action consistent with the data was CNS outside the blood-brain barrier. With low infusion rate, most of the total brain AChE was inhibited. With increasing infusion rate, inhibition of total brain AChE activity would decrease, due to less time for paraoxon to penetrate the BBB; the extra-BBB site would always be rapidly inhibited.

Also measured was AChE in the brain, heart and diaphragm after infusion of sublethal doses of paraoxon at various rates, Figures 2 and 3. Heart and diaphragm AChE was at the level observed in CoB while inhibition of brain AChE increased with increasing dose, again indicating brain as the sensitive site.

FUTURE WORK

With much of the necessary methodology in place, we will pursue collection of data in both rat and fish:

1. The infusion experiment will be repeated in rainbow trout, to locate the site of action of paraoxon in that species. Rather than anesthetize the trout during the paraoxon infusion, we plan to sever the spinal cord below the head after the aortic cannula is placed under anesthesia. With this preparation, the fish breathes normally; under anesthesia, they do not and it would therefore not be possible to use the same death end point as in the rat.
2. For one of the intermediate infusion rates, we will terminate the infusion and sample tissues for paraoxon and AChE determination at 20%, 40%, 60% and 80% of the time required for death. This experiment will provide information to construct a graph of AChE inhibition vs plasma concentration of paraoxon for each tissue studied. A mathematical relationship will be sought and if obtained it will be used in the development of a pharmacokinetic-pharmacodynamic model for paraoxon.
3. Develop a pharmacokinetic-pharmacodynamic model for paraoxon in fish and rat. Some pharmacokinetic modeling of paraoxon in rat and dog has been published (Eigenberg, Pazdernik and Doull, Drug Metabolism and Disposition 11:366,1983.) The reported half-life in rat is very short (3.3 min) and the volume of distribution large (2.2 liters/kg). We will administer paraoxon as an infusion to truncate the distributive phase and attempt to characterize the post-infusion plasma disappearance curve. Because fish are much less sensitive to the AChE inhibition effect of paraoxon than are mammals, it will be possible to use higher doses and thereby make it possible to follow the kinetics for a longer period of time post infusion. Using tissue AChE activity vs paraoxon concentration relationships from 2. and the concentration-time data from this part, we will work to develop a mathematical model that predicts paraoxon concentration vs time and AChE inhibition vs time after any route of administration.

PUBLICATIONS

Development of methods consumed much of this project period and we have not yet collected enough data on which to base a research paper. The AChE-infusion rate data have been submitted for presentation at the November annual meeting of the Society for Environmental Toxicology and Chemistry.

The following papers from our lab have appeared or been submitted during the project period:

- A.H. Karara and W.L. Hayton. Metabolic Inhibition and Di-2- Ethylhexyl Phthalate Pharmacokinetics in Fish. Drug Metab. Disp. 16:146-150 (1988).
- A.H. Karara and W.L. Hayton. A Pharmacokinetic Analysis of the Effect of Temperature on the Accumulation of Di-2-Ethylhexyl Phthalate (DEHP) in Sheepshead Minnow. Aquatic Toxicology 15:27-36 (1989).
- G.R. Stehly and W.L. Hayton. Errors in the Use of the Accelerated Bioconcentration Test. Accepted for publication in the Proceedings of the ASTM Aquatic Toxicology Series.
- G.R. Stehly and W.L. Hayton. Detection of Pentachlorophenol and its Glucuronide and Sulfate Conjugates in Fish Bile and Exposure Water. J Environm Sci and Hlth, Part B. B23:355-366 (1988).
- M.G. Barron, I.R. Schultz and W.L. Hayton. Presystemic Branchial Metabolism: Limits Di-2-ethylhexyl Phthalate Accumulation in Fish. Toxicol. Appl. Pharmacol. 98:49-57(1989).

- G.R. Stehly and W.L. Hayton. Metabolism of Pentachlorophenol by Fish. *Xenobiotica* 19:75-81(1989).
- M.S. Dey, R.G. Breeze, W.L. Hayton, A.H. Karara and R.I. Krieger. Paraquat Pharmacokinetics Using a Subcutaneous Toxic Low Dose in the Rat. *Fund. Appl. Toxicol.* 14:208-216 (1990).
- W.L. Hayton and M.G. Barron. Rate Limiting Barriers to Xenobiotic Uptake by the Fish Gill. *Environ. Toxicol. Chem.* 9:151-157 (1990).
- G.R. Stehly and W.L. Hayton. Effect of pH on the Accumulation Kinetics of Pentachlorophenol in Goldfish. *Arch. Environm. Contam. Toxicol.* 19:464-470 (1990).
- W.L. Hayton. Interspecies Scaling Pharmacokinetic Models. *Health Physics* 57: Suppl. 1, 159-164 (1989).
- G.R. Stehly and W.L. Hayton. Disposition of Pentachlorophenol in Rainbow Trout (*Salmo gairdneri*): Effect of Inhibition of Metabolism. *Aquatic Toxicology* 14:131-148(1989).
- B.D. Tarr, M.G. Barron and W.L. Hayton. Effect of Body Size on the Uptake and Bioconcentration of Di-2-Ethylhexyl Phthalate in Rainbow Trout. Accepted for *Environmental Toxicology and Chemistry*
- M. Yokokawa, R. Nishigaki, K. Umemura and W.L. Hayton. Intestinal Absorption Kinetics Using a Laminar Flow Model. *J. Pharmacobiodyn.* 12:332-340(1989).
- W.L. Hayton, J. Kneer, R.A. Blouin and K. Stoeckel. Pharmacokinetics of Intravenous Cefetamet and Oral Cefetamet Pivoxil in Patients with Hepatic Cirrhosis. Accepted for *Antimicrob. Agents Chemother.*
- W.L. Hayton, V. Vlahov, N. Bacracneva, I. Viachki, R. Portmann, J. Muirhead, K. Stoeckel and E. Weidekamm. Pharmacokinetics and Biliary Concentrations of Fleroxacin in Cholecystectomized Patients. submitted to *Antimicrob. Agents Chemother.*
- S.S. Narayan, W.L. Hayton and G.S. Yost. Chronic Ethanol Consumption Causes Increased Glucuronidation of Morphine in Rabbits. Accepted for *Xenobiotica*.
- M.G. Barron, G.R. Stehly and W.L. Hayton. Pharmacokinetic Modeling in Aquatic Animals. Accepted for *Aquatic Toxicology*.
- W.L. Hayton and I.R. Schuitz. Scaling Bioconcentration and Pharmacokinetic Parameters for Body Size and Environmental Variables in Fish. presented at, and submitted to Proceedings of, the 14th Symposium on Aquatic Toxicology and Risk Assessment. American Society for Testing and Materials, April, 1990.
- I.R. Schuitz and W.L. Hayton. Experimental Design for Pharmacokinetic Data Analysis Using Compartmental Pharmacokinetic Models. presented at, and submitted to Proceedings of, the 14th Symposium on Aquatic Toxicology and Risk Assessment, American Society for Testing and Materials, April, 1990.
- W.L. Hayton, J. Kneer and K. Stoeckel. Pharmacokinetics of Intravenous Cefetamet and Oral Cefetamet Pivoxil in Children. submitted to *Antimicrob. Agents Chemother.*
- W.L. Hayton. Symbol-and-Arrow Diagrams In Teaching Pharmacokinetics. *A.m. J. Pharm. Ed.*, in press.

Figure 1. Percent inhibition of acetylcholinesterase in rat brain (■), heart (□), and diaphragm (◆) versus rate of infusion of paraoxon.

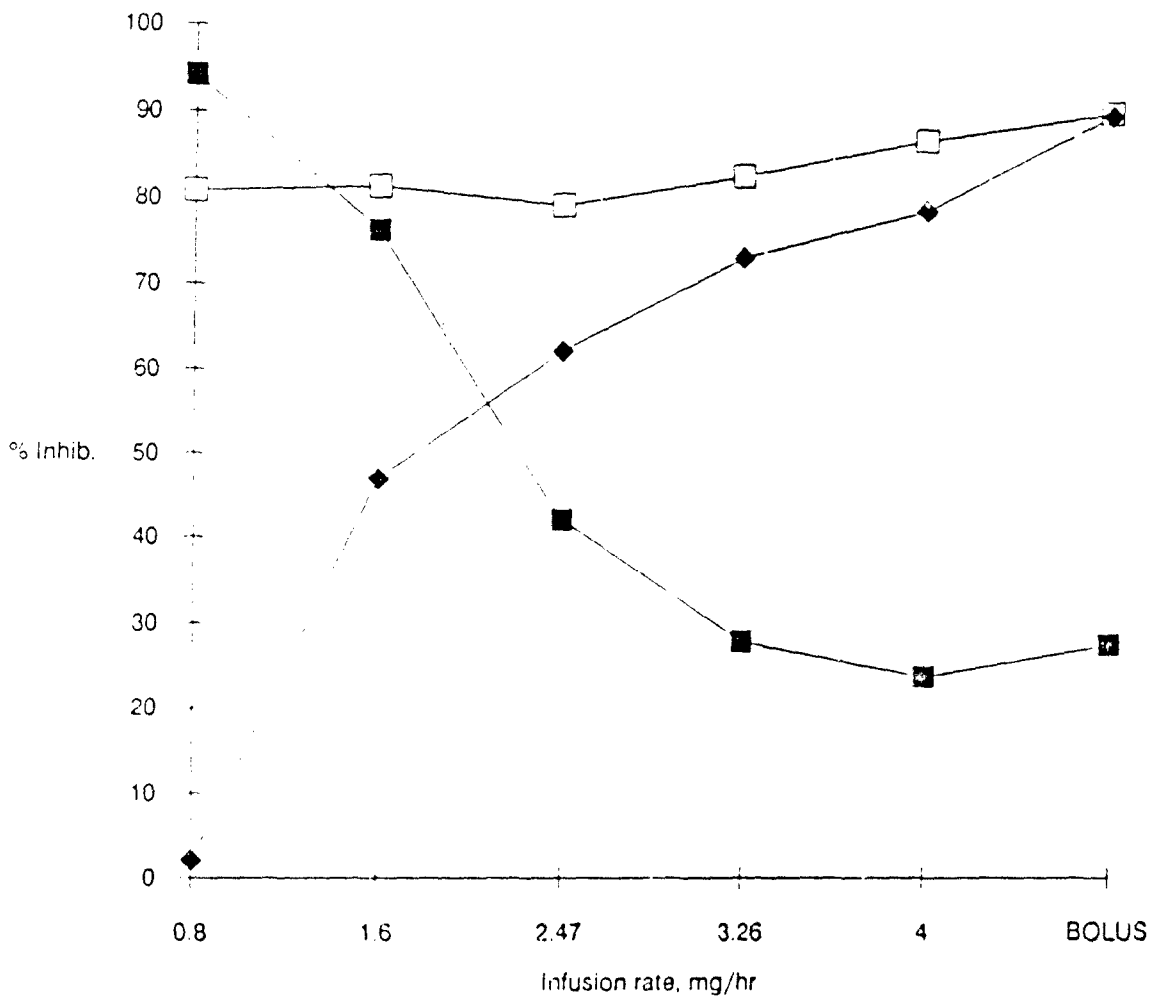


Figure 2. Percent inhibition of acetylcholinesterase in rat brain (■), heart (□), and diaphragm (◆) after infusion of various doses of paraoxon at an infusion rate of 3.26 mg/hr.

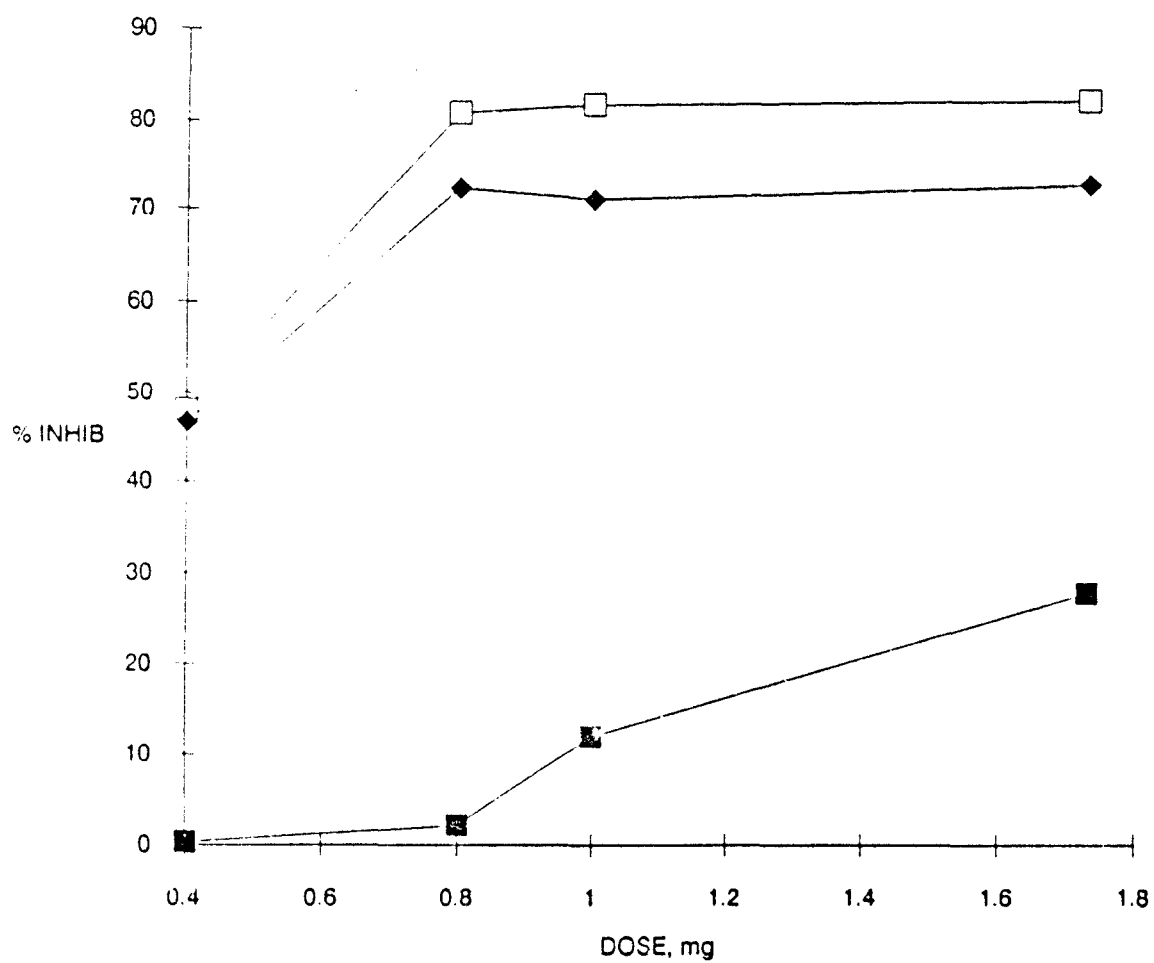
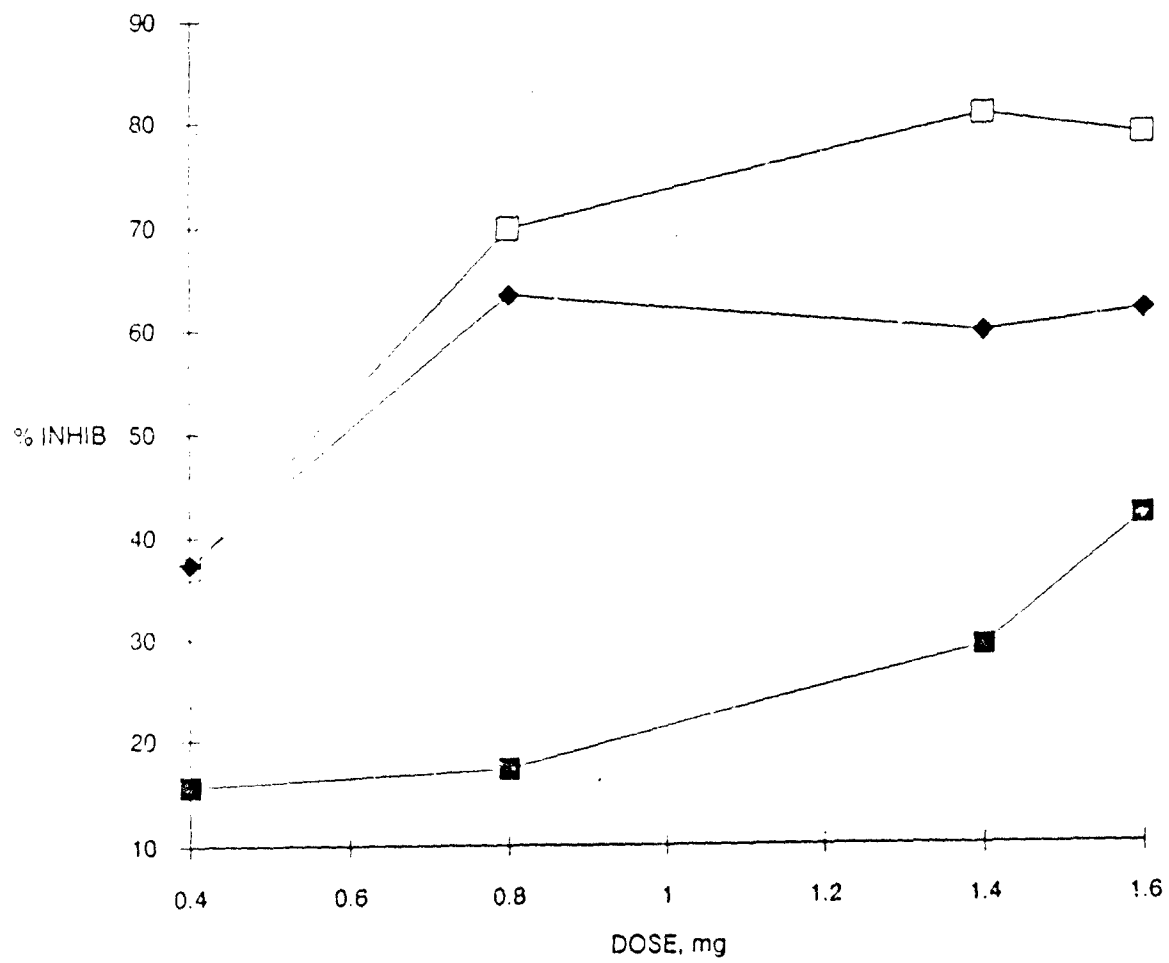


Figure 3. Percent inhibition of acetylcholinesterase in rat: brain (■), heart (□), and diaphragm (◆) after infusion of various doses of paraoxon at an infusion rate of 2.47 mg/hr.



Abstract submitted to 1990 annual meeting of the
society for Environmental Toxicology and Chemistry:

**EFFECT OF PARAOXON INFUSION RATE ON AChE
CONCENTRATION IN RAT BRAIN, HEART AND
DIAPHRAGM. W.L. Hayton and U. Herzberg, Coll. of
Pharmacy, Wash. St. Univ., Pullman, WA USA.**

Paraoxon inhibits acetylcholinesterase (AChE) to cause death. While most tissues contain AChE, the site at which AChE inhibition results in death is unknown. To identify the sensitive site, paraoxon was infused into the jugular vein of pentobarbital anesthetized Fisher 344 male rats at rates from 0.8 to 4.0 mg/hr. At cessation of breathing (CoB) samples of brain, heart and diaphragm were removed and AChE determined using the Ellman method. At all inf. rates, heart AChE was inhibited 80%; with increasing inf. rate inhibition of brain AChE decreased (95% to 20%) while inhibition of diaphragm AChE increased (2% to 90%). The dose of paraoxon at CoB averaged 5.7 mg/kg at all inf. rates. While heart AChE activity at CoB was independent of the inf. rate, heart appeared not to be the sensitive site since it was pumping blood at CoB. A site of action consistent with the data was CNS outside the blood-brain barrier. With low inf. rate, most of the total brain AChE was inhibited. With increasing inf. rate, inhibition of total brain AChE activity would decrease, due to less time for paraoxon to penetrate the BBB; the extra-BBB site would always be rapidly inhibited. Also measured was AChE in the brain, heart and diaphragm after inf. of sublethal doses of paraoxon at various rates. Heart and diaphragm AChE was at the level observed at CoB while inhibition of brain AChE increased with increasing dose, indicating brain as the sensitive site. (Supported by AFOSR-88-0345).