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ACTIVATION OF THE PHOSPHOROTHIONATE INSECTICIDE PARATHION BY RAT BRAIN IN SITU

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The ability of the rat brain to activate the phosphorothionate insecticide parathion to its potent anticholinesterase metabolite paraoxon in situ was observed by ligating the posterior portion of the circulatory system and thus removing the liver from the circulation. Under these conditions no acetylcholinesterase inhibition was observed in 15 min at a dosage of parathion (nominally 2.4 mg/kg) which yielded 95% inhibition when the liver was in the circulation. However, at a higher dose (nominally 48 mg/kg) there was substantial (about 70%) inhibition of brain acetylcholinesterase after 15 min, suggesting that the brain does have the ability to activate parathion in the intact situation.

Phosphorothionate insecticides, such as parathion, are activated to potent anticholinesterases, their oxon metabolites, by a cytochrome P-450-mediated desulfuration reaction (1). This activation decreases the I_{50} to the target enzyme, acetylcholinesterase, by about 3 orders of magnitude (2,3). The oxons are also very potent inhibitors of other serine esterases, and they are usually more potent inhibitors of hepatic aliesterases than of brain acetylcholinesterase (2). This strong affinity for aliesterases suggests that hepatically-generated oxon molecules would readily phosphorylate aliesterases and other susceptible proteins in the liver and the blood. This behavior may contribute substantially to the covalent binding of parathion or its metabolites observed in the perfused rat liver (4). A-Esterase-mediated hydrolysis could also detoxify the oxon (3,5). Thus, a large proportion of the oxon molecules formed in the liver could be destroyed before they have an opportunity to reach the target acetylcholinesterase in the brain.

The importance of extrahepatic activation of parathion has been suggested (6,7). We have reported in vitro activation of six phosphorothionates by both microsomal and crude mitochondrial fractions of rat brain; this activity was low compared to the activity of hepatic microsomes (2,3,8).

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However, the question still remained of whether this monooxygenase activity contributes any oxon in the intact animal. These experiments were designed to determine whether brain acetylcholinesterase becomes inhibited in an intact organism following administration of the phosphorothionate insecticide parathion after the liver had been removed from the circulation. Following ligation of the posterior portion of the circulation and administration of parathion into the jugular vein, the extent of brain acetylcholinesterase inhibition was determined as an index of extrahepatic paraoxon formation.

METHODS

Animals: Male Sprague-Dawley derived rats [Crl:CD(SD)BR] from an original Charles River stock weighing between 525-765 g were used. Before use they were housed in a temperature-controlled animal room with a 12:12 hr light cycle and with Purina laboratory rodent food and water freely available.

Chemicals: Parathion was a gift of Monsanto Chemical Company (St. Louis, MO) and was purified by recrystallization from methanol. All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Surgical procedure and treatment: All rats were anesthetized with diethyl ether to prevent cardiovascular and respiratory depression and were maintained throughout experimentation under ether anesthesia; all procedures were conducted in a fume hood. In rats which were to have the liver removed from the circulation (liver-ligated), the aorta was ligated immediately posterior to its exit from the diaphragm. A small (30-35 mg) section of liver (pre-injection sample) was taken immediately before ligation for aliesterase assay and was frozen at -70°C. The liver-intact animals were sham-operated, and no pre-injection liver sample was taken.

Following abdominal surgery, the abdominal skin was replaced and the opening was covered with a paper wipe moistened with saline to prevent tissue drying. The jugular vein was exposed and parathion dissolved in propylene glycol was injected using a 27 gauge needle. The injection itself took 45 sec. The needle was left in place for an additional 15 sec to insure that the injection solution flowed past the injection site. Propylene glycol alone was injected into the controls in the same manner.

Animals were sacrificed at either a nominal "0 min" or 15 min after injection. The 0-min animals were sacrificed within 60-90 seconds following completion of the injection; thus sufficient time was allowed for the injection solution to enter the circulation and to distribute. Immediately before euthanasia a section of liver (post-injection sample) was taken and frozen for subsequent aliesterase assay. Animals were decapitated and the corpus striatum, medulla oblongata, and a section of the posterior cerebral cortex were removed for acetylcholinesterase analysis. Brain samples were either assayed immediately or frozen at -70°C for no more than 4 days prior to analysis.

Dosage: An initial dosage of parathion was selected which yielded about 95% brain acetylcholinesterase inhibition following a 15 min incubation with the liver intact. This dosage was 2.4 mg/kg administered in 360 μl/kg, so that approximately 200 μl was injected per animal. Parathion yielded a very steep dose-response curve with initial studies indicating that 2.25 mg/kg resulted in only 20% inhibition. Using an estimate that 60% of the circulatory volume had been removed by ligation, 40% of this dosage was administered to the liver-ligated animals.

A higher dose of parathion was also tested in liver-ligated animals. In this case 360 μl/kg of a 52 mg/ml solution of parathion in propylene glycol (the solubility limit) was administered, equivalent to 19 mg/kg (although the dosage only circulated in the anterior region of the animal). The design was to administer the highest dose possible without exceeding an injection volume of about 200 μl. Considering the estimate of 40% blood volume remaining in the circulation, this dosage is nominally 48 mg/kg in the anterior portion of the animal.

Acetylcholinesterase assay: Acetylcholinesterase activity was assessed using a modification of the Ellman et al. (9) technique, as previously described (10). The concentrations used were: cerebral cortex, 1 mg/ml; corpus striatum, 0.125 mg/ml; and medulla oblongata, 0.833 mg/ml. Protein was quantitated using bovine serum albumin as the standard (11).

Aliesterases: Aliesterases were assayed in liver samples to determine whether oxon was present in the liver. The information was used to guarantee that the liver was not part of the circulation in the liver-ligated animals treated with parathion. In addition, pre- and post-injection samples in untreated animals were used to assure that removal of the liver from the circulation for 15 min did not cause deterioration of aliesterase activity. Liver was homogenized in 0.25 M sucrose at 50 mg/ml and centrifuged at 1000 g for 15 min. The supernatant was used following dilution with 0.1 M Tris-HCl buffer, pH 7.4, to a concentration of 0.05 mg equivalents/ml. Four ml of the diluted tissue suspension were temperature equilibrated to 37°C in a shaking water bath. After 15 min equilibration, 40 μl of a 0.05 M 4-nitrophenyl valerate solution in ethanol was added, and incubation proceeded for 15 min. The reaction was terminated by addition of 1 ml of a solution of 2% sodium dodecyl sulfate plus 2% Tris base. Absorbance was monitored at 400 nm. Subsamples were run in triplicate. Non-enzymic hydrolysis was assessed in parallel samples inhibited during the equilibration period with 10^{-5} M paraoxon.

RESULTS

Acetylcholinesterase activity was unaffected by propylene glycol or by either dose of parathion at "0 min" in any brain region (Table 1). At the low parathion dose, the acetylcholinesterase activities were not depressed at 15 min when the liver was ligated, but they were inhibited about 95% in the intact system. At the high dose there was a significant inhibition (67-74%) of acetylcholinesterase after 15 min in all three brain regions.

Hepatic aliesterase activity was unchanged by any treatment at any time except in the liver-intact group at 15 min after parathion treatment (Table 2).

DISCUSSION

The *in vitro* presence of phosphorothionate desulfuration activity in preparations of rat brain (2,3) does not prove that these monooxygenases are capable of generating product *in vivo*, especially considering their low activities. The experimental design used here guaranteed that the liver was not contributing paraoxon. The fact that hepatic aliesterases were not inhibited after 15 min in the liver-ligated groups (both high and low doses)

Table 1

Effect of parathion on rat brain acetylcholinesterase activity in the presence or absence of the liver in the circulation

Liver Surgical Condition	Injection	Time, min	Specific Activity[1] Cerebral cortex	Corpus striatum	Medulla oblongata
Intact	Control	--	58.59±2.68(4)	454.7±22.5(4)	85.81±1.63(4)
	Vehicle	15	55.56±2.78(3)	419.4±35.6(3)	80.13±0.67(3)
	Parathion, 2.4 mg/kg	0	58.00±3.46(4)	453.1± 7.7(4)	86.92±1.74(4)
		15	2.99±0.99(5)*	23.1±12.0(5)*	7.02±3.34(5)*
Ligated	Vehicle	15	51.56±1.99(3)	467.0±33.7(3)	87.62±4.66(3)
	Parathion, 2.4 mg/kg	0	50.76±8.83(4)	392.7±53.8(4)	72.93±8.27(4)
		15	48.78±1.67(5)	396.3±21.8(5)	71.40±3.70(5)
	Parathion, 48 mg/kg	0	44.22±3.48(5)	393.4±21.4(5)	69.46±5.10(5)
		15	14.70±2.18(4)*	150.2±29.8(4)*	26.58±3.32(4)*

[1]Data are expressed as nmoles product formed/min/mg product, mean±S.E.M. (N).
*Mean is significantly different from control mean by SNK ($P<0.05$).

clearly indicated that the liver had been removed from the circulation. Parathion itself is capable of inhibiting acetylcholinesterase, but at a potency about 3 orders of magnitude lower than paraoxon (3). The 0-min acetylcholinesterase activities, being the same as control activities, gave assurance that any acetylcholinesterase inhibition observed in the experiment

Table 2

Effect of parathion on rat liver aliesterase activity in the presence or absence of the liver in the circulation

Injection	Time, min	Specific Activity[1] Intact	Ligated Pre-injection	Ligated Post-Injection
Control	--	534.4± 34.7(4)	--	--
Vehicle	15	349.5± 39.7(3)	365.5±42.9(3)	358.3±44.5(3)
Parathion, 2.4 mg/kg	0	590.6±238.8(4)	490.6±97.1(4)	420.2±80.4(4)
	15	30.8± 5.7(5)*	652.3±78.5(5)	608.7±74.2(5)
Parathion, 48 mg/kg	0	--	475.8±58.9(5)	408.8±94.3(5)
	15	--	516.5±81.5(5)	505.7±83.1(5)

[1]Data are expressed as nmoles/min/mg protein, mean±S.E.M. (N).
*Mean is significantly different from control mean by SNK ($P<0.05$).

was not merely the result of high levels of parathion in the brain or its circulation.

The design of the low dose experiment was to yield a substantial but not saturating level of paraoxon inhibition in the brain in the hope that at least a small fraction of the paraoxon generated had been formed in the brain. Under these conditions, the brain did not appear to be responsible for detectable levels of paraoxon. At these levels of circulating parathion, in the liver-intact animals, the liver was probably capable of efficiently extracting most or all of the parathion from the blood, and allowing little to flow back into the circulation. Others have reported that the rat liver readily absorbs parathion from the blood (4,12). It has been demonstrated that both methyl parathion and parathion and their oxons occur in the effluent of perfused mouse liver (13,14), with the phosphorothionate occurring in much higher concentrations than the oxon. If the dose used here was at the appropriate level, the liver would have effectively extracted the parathion from the circulation and returned both parathion and paraoxon to the circulation, with the hepatically-generated oxon then responsible for virtually all brain acetylcholinesterase inhibition. Clearly, the above scenario would not have occurred in the liver-ligated rats. Apparently at these levels of substrate the brain monooxygenases were unable to generate sufficient paraoxon quickly enough to affect brain acetylcholinesterase significantly.

The dosage used in the above experiments, even though the low dosage in our protocols, was still a very high dose that would probably have been lethal in a short period of time. Experiments in our laboratory with parathion have indicated that 15 mg/kg i.p. is lethal at 90 min and 1.3 mg/kg of paraoxon i.p. is lethal within 4 hr. Thus, this dose delivered intravenously was probably reflective of a severe acute poisoning. In such a situation, in which the organophosphate levels are high and exceed the capacity of the liver to retain phosphorothionate and oxon alike, the liver probably contributes essentially all of the oxon which poisons the brain.

In light of these results, we attempted a different protocol to demonstrate desulfuration activity in the brain. Because the brain parathion desulfuration activity is present at a very low level (about 19-fold less than in liver in male rats; 3), there were 2 options for enhancing metabolite production, i.e., increasing either the time or the substrate concentration. The former option was deemed undesirable because it was unlikely that the circulation could have been maintained and the tissues kept viable in a liver-ligated rat for the 1-2 hr probably required to generate measurable amounts of oxon. Therefore, it was decided to use a 15-min incubation time, in which the liver-ligated rats could be confidently maintained, and to increase the parathion levels to the highest dose that could be administered in a

reasonable injection volume. The dosage selected is admittedly well above that experienced in a severe accidental poisoning. However, with sufficient substrate available, very substantial brain acetylcholinesterase inhibition occurred, indicating that the brain monooxygenases were capable of generating oxon in an in situ situation. Although it is possible that other organs in the anterior portion of the animal could have generated the paraoxon, this seems unlikely. Lung parathion desulfuration activity is low (6), and other tissues left in the circulation would also be expected to be of low activity. Additionally, the aliesterases of rat plasma are readily inhibited by paraoxon (unpublished data) and would serve as an alternate phosphorylation site (and therefore degradation site) for oxon released by any of the other tissues. Therefore, it is felt that the acetylcholinesterase inhibition observed in the high dose, liver-ligated animals arose from paraoxon generated by the brain monooxygenases.

Thus, in the intact animal parathion desulfuration can occur in the brain in close proximity to the target acetylcholinesterase. Although this activity probably contributes little in a severe acute poisoning where the liver is releasing relatively large amounts of paraoxon into the circulation, it may well be of significance in a lower dose repeated or continuous exposure, such as occurs in most occupational settings, where there is first pass entry into the brain from dermal contact and where the protective mechanisms in the liver are functioning to retain and/or detoxify all of the generated paraoxon.

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