Inhibition of Rat Cholinesterases by Tritolyl Phosphates.* (23574)

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Triorthotolyl phosphate has been of considerable interest to pharmacologists because of its ability to produce flaccid paralysis in fowl and in some mammalian species. The neurotoxic mechanism is obscure, and at present cholinesterase inhibition is not believed to be involved(1,2). It has been established that the pure compound does not inhibit cholinesterase *in vitro* but does inhibit plasma cholinesterase in rabbits on intravenous injection(3) and in fowl(4). The effect on erythrocyte cholinesterase of mammals has not been investigated previously, so far as we know. In this paper we report results of studies on inhibition of rat cholinesterases after intraperitoneal injection of 3 isomers of tritolyl phosphate.

Procedures. Triorthotolyl phosphate (TOTP), trimetatolyl phosphate (TMTP) and triparatolyl phosphate (TPTP) were obtained from the Eastman Organic Chemicals, Rochester, N. Y. Infra-red and mass spectroscopy indicated 99% purity, with the remaining 1% free of possible inhibitory agents. No ortho-groups were present in the samples of TMTP and TPTP, and the TOTP contained no pyro-groups. Male rats of the Long-Evans strain were used. For single-dose studies, their weights ranged from 85 to 110 g; for repeated-dose studies, the initial weights ranged from 120 to 150 g. The rats were randomized into groups for treatment, and were withdrawn from the repeated-dose experiment by random selection. *Single injection.* Groups of 5 rats were given 2 ml/kg of TOTP intraperitoneally and decapitated after intervals of 1, 12, 24, 48, and 168 hours. Groups of 5 rats were given 2 ml/kg of TMTP and TPTP intraperitoneally and decapitated after 24 hours. Groups of 5 uninjected control rats were sacrificed with the experimental groups in each case. TOTP and TMTP were given undiluted, while TPTP was given as a 10% emulsion in distilled water. The activity of cholinesterases in the erythrocytes, plasma, and brain was determined by the method of Frawley(5) and calculated as percentage with reference to the control groups. *Repeated injection.* The effect of repeated sublethal doses of TOTP was studied by injecting 2 groups of 10 rats with 0.1 and 0.5 ml/kg of TOTP intraperitoneally, thrice weekly, as 20 and 95% suspensions in propylene glycol, respectively. Control rats received repeated injections of 0.04 ml of propylene glycol, an amount equal to the largest quantity given the experimental animals. After sixth injection (on twelfth day), 5 rats given each level of TOTP and 5 controls were sacrificed for determination of cholinesterase activity in blood cells, plasma, and brain. Sections of liver, spinal cord, and sciatic nerve were retained for histologic examination. The remaining 5 rats in each group were sacrificed 10 days after the sixth injection, for cholinesterase determination and histologic study as above.

Results. Single injection. After a single intraperitoneal injection of 2 ml/kg TOTP, cholinesterase activity became increasingly less, to reach the lowest recorded value at 48 hours (Table I). At all intervals after the first hour, the erythrocyte enzyme was least active. Signs of acetylcholine accumulation were first seen 24 hours after injection, and were more severe after 48 hours. These signs were seen only in individuals with cholinesterase activity of less than 20% in the brain, erythrocytes, or both. They consisted of diarrhea, champing of teeth, muscle tremors including shaking paws, and occasional tear formation.

Rats sacrificed one hour after injection

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TABLE I. % Active Cholinesterase in Rat Tissues after a Single Intraperitoneal Injection of 2 ml/kg TOTP.

Hr after inj.	Enzyme source: Erythrocyte	Plasma	Brain
1	90.7	66.3	88.0
12	30.2	41.9	38.5
24	14.2	38.6	19.5
48	4.0	31.7	10.8
168	44.8	69.8	48.2

showed no gross abnormalities. A slight greasy appearance of the abdominal organs indicated incomplete absorption of the TOTP. Rats sacrificed at 12 hours had swollen livers with white mucoid material along their edges. Unabsorbed TOTP was still present in the cavity. In rats sacrificed at 24 and 48 hours there was a white mucoid material along the edges of the swollen livers, but no observable TOTP remained in the cavity. Seven days after injection, swelling was still present in the livers of half the rats, and the lobes were adherent. Small patches of white mucoid matter appeared to be contained within the liver tissue. The other rats of the group showed no abnormalities.

Microscopically, the animals showed a peritonitis of varying degrees of chronicity, depending on length of survival. The livers showed no parenchymal changes.

There were marked differences in the effects of the 3 isomers 24 hours after injection. TOTP lowered cholinesterase activity of all 3 tissues significantly (Table I) while TMTP significantly lowered only the plasma enzyme (60% of normal). TPTP had no significant effect on cholinesterase. TMTP and TPTP as well as TOTP had been completely absorbed by this time. No abnormalities were seen in rats injected with TMTP, and none in those injected with TPTP except for a few nodules scattered throughout the abdominal cavities of 2 rats.

Repeated injection. When repeated sublethal injections were given, TOTP again inhibited the true cholinesterase to a greater extent than the pseudocholinesterase. After 6 injection of 0.1 ml/kg, the cholinesterase of the erythrocytes was only 27% active while that of the plasma was 67% active; that of the brain was 47% active. With a 5-fold increase in the dose, the activity of cells or plasma was not significantly less than with the lower dose, but that of the brain was halved. None of the animals showed clinical signs of cholinesterase inhibition, presumably because the decrease did not pass the critical point. All of these animals had swollen livers with small white mucoid areas on the surfaces. Rats receiving the larger dose had in addition, small semi-solid lumps scattered through the omentum. The control rats were essentially normal.

The rats sacrificed 10 days later still had significantly lowered true cholinesterase values. The erythrocyte cholinesterase of the rats receiving 0.1 ml/kg was 67%, the brain was 55% active, and the plasma was up to normal, 97%. At the higher dose of 0.5 ml, the erythrocyte and brain values were somewhat lower, 53% and 38%, while the plasma was 80% active. These figures show that the activity of the brain enzyme is the slowest to be recovered, and suggest that it may be the most profoundly affected. These rats showed no clinical signs of cholinesterase inhibition, and no lesions except for swollen livers with patches of white mucoid material.

Microscopically, many of the livers exhibited a foreign-body reaction, a local effect of the compound which was not seen in the propylene glycol controls. The liver parenchyma was normal in all cases, and the spinal cords showed no difference between experimental animals and controls.

Discussion. It has been widely stated that TOTP is a specific inhibitor of pseudocholinesterase, and does not inhibit true cholinesterase. This belief apparently stems from a report of Mendel and Rudney(6) which stated that rats fed on the compound showed lowered plasma cholinesterase but normal erythrocyte cholinesterase. A complete absence of effect would have been expected, in view of the report of Smith *et al.*(7) that administration of TOTP to albino rats by various routes in doses as high as 30 g/kg produced no response, except for pulmonary edema after intravenous injection. They concluded that the albino rat was wholly refractory.

In the rabbit, however, severe inhibition of

true cholinesterase is suggested by their report(8) of muscular tremors and other signs attributable to accumulation of acetylcholine, following peroral administration. The guinea pig showed similar signs, while the dog and monkey did not appear to absorb the compound very well from the alimentary canal.

The possibility that true cholinesterase might be inhibited, even in the rat, was suggested by some of our earlier (unpublished) experimental work. We found typical muscular signs following administration of TOTP to rats of the Long-Evans strain. We do not know why our results should differ from those of Smith and his associates. However, the results of this experiment have demonstrated an inhibition of erythrocyte cholinesterase in this strain which is even more profound than the inhibition of plasma cholinesterase.

The only other relevant investigation which we have knowledge of is a brief experiment by Aldridge(3). He injected rabbits intravenously with 6.8 mg/kg TOTP and found 81% inhibition of plasma cholinesterase after 30 minutes, but only 58% inhibition of erythrocyte cholinesterase at 90 minutes. From our results with rats, it seems likely that inhibition of erythrocyte cholinesterase would have been much greater if the blood had been tested in 12 to 24 hours.

Aldridge demonstrated in the same paper that TOTP must be metabolized before cholinesterase inhibition occurs. If different metabolites are involved in the two types of inhibition, this might account for the delayed inhibition of erythrocyte cholinesterase. However, the time difference might also be attributable to slow penetration of the red blood cell.

The results obtained with the 2 isomers of TOTP were almost as anticipated. It was not expected that either would cause any cholinesterase inhibition, and although trimetatolyl phosphate did reduce plasma activity, no other effects were noted.

Summary. 1) In contrast to previous reports, TOTP was found to be an active inhibitor of true cholinesterase in erythrocytes and brain of rats. Cholinergic signs were noted when activity in either tissue fell to less than 20% of normal. Maximum inhibition occurred 24 hours after single injection and exceeded inhibition of the plasma enzyme. Repeated sublethal doses also produced greater inhibition of true than of pseudocholinesterase. 2) On the other hand, the plasma enzyme was inhibited 24 hours after a single dose of TMTP while the cholinesterase of erythrocytes and brain was not significantly affected. TPTP failed to inhibit the enzyme of any of the 3 tissues. 3) No signs of paralysis were elicited, and no histologic lesions were discovered in the peripheral or central nervous system.

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