Investigation of Multiple Mechanisms for Potentiation of Malaoxon's Anticholinesterase Action by Triorthotolyl Phosphate[1] (36899)

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The acute toxicity of the organophosphate insecticide malathion [*O,O*-dimethyl *S*-(1, 2-dicarbethoxyethyl) phosphorodithioate] results from acetylcholinesterase inhibition following *in vivo* oxidation to its anticholinesterase metabolite, malaoxon [*O,O*-dimethyl *S*-(1,2-dicarbethoxyethyl) phosphorothiolate] (1, 2). Both malathion and malaoxon are hydrolytically detoxified by tissue carboxylesterases (EC 3.1.1.1) to metabolites which are not cholinesterase inhibitors (3, 4). Inhibition of carboxylesterase activity by triorthotolyl phosphate (TOTP) greatly increases the susceptibility of mice to malathion poisoning (4, 5). A close association has been demonstrated between the degree of potentiation of malathion toxicity and inhibition of liver carboxylesterase activity by low doses of TOTP. However, the degree of malathion potentiation continued to increase with increasing doses of TOTP beyond the dose which caused maximum inhibition of carboxylesterase activity (6, 7). In the present investigation, a similar phenomenon was observed for the relationship between degree of potentiation of malaoxon toxicity and inhibition of carboxylesterase activity by TOTP. This suggested that another mechanism, in addition to carboxylesterase inhibition, might be involved in the potentiation of malathion and malaoxon by TOTP. This investigation was undertaken to study possible mechanisms for the potentiation observed after the higher doses of TOTP.

Materials and Methods. Male mice (Charles River, CD-1) weighing 20 to 30 g were used. They were housed in air-conditioned rooms (70–80°F) and were supplied with food and water *ad libitum*. TOTP (Practical grade) and malaoxon (95%) were injected, ip as corn oil solutions. The concentrations of the solutions were adjusted to provide the required dose in an injection volume of 5 ml/kg. Control mice were given only corn oil.

Mice were sacrificed by decapitation and exsanguination. Whole brains were homogenized in calcium-free Ringer–bicarbonate buffer, pH 7.6. Other tissues were homogenized in 0.026 *M* sodium bicarbonate buffer, pH 7.6. Homogenates were stored at 0–4° until the time of assay and all assays were completed within 24 hr after the animals were sacrificed.

Brain cholinesterase activities were assayed manometrically with 50 mg of brain (8). Acetylcholine chloride (0.01 *M*) was used as the substrate. Liver, lung and plasma cholinesterase activities were determined colorimetrically (9) with 2, 3 and 10 mg of tissue, respectively. Acetylthiocholine iodide (0.001 *M*) was used as the substrate. Mean ± SE cholinesterase activities from 5 to 10 control mice were 10.1 ± 0.1, 3.5 ± 0.3, 2.2 ± 0.1, and 1.3 ± 0.1 μmole of substrate hydrolyzed/min/g of brain, liver, lung and plasma, respectively.

For assays of carboxylesterase activity, hydrolysis of 0.0067 *M* diethyl succinate by 2.5 mg, 0.027 *M* triacetin by 5.0 mg and 0.0067 *M* malathion by 200 mg of homogenized liver and 0.027 *M* triacetin by 150 mg of plasma was determined manometrically as described previously (7, 8). Mean ± SE carboxylesterase activities of 5 to 10 control mice were

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5136 ± 165, 3601 ± 60, and 55 ± 2 μl CO_2/g/min for liver hydrolysis of diethyl-succinate, triacetin and malathion, respectively, and 55 ± 3 μl CO_2/g/min for plasma hydrolysis of triacetin. In all cases, tissue levels and substrate concentrations were selected to provide the optimum conditions at which enzyme activity of the tissue would be the rate-limiting factor in the assay.

Malaoxon binding by mouse liver was determined by measuring the loss of anti-cholinesterase activity of a known quantity of malaoxon (20 nmoles) incubated with 50 mg of homogenized liver for 1 min at 1° in 5 ml (total volume) of 0.02 *M* phosphate buffer, pH 7.6. Aliquots of the incubate were added to 0.39 μ*M* units of bovine erythrocyte cholinesterase (Sigma Chemical) in a 3.0 ml calcium-free Ringer–bicarbonate buffer system containing 0.01 *M* acetycholine chloride as the substrate. Malaoxon content of the aliquots was determined from a standard curve for inhibition of cholinesterase by malaoxon as described previously (10) and was compared with the malaoxon content of a malaoxon blank which was carried through the same procedure. Malaoxon binding was calculated as the difference between the amount of malaoxon remaining in the liver incubate and in the blank. Mean ± SE malaoxon binding activity of 8 control livers was 280 ± 10 nmoles/g of liver.

Results. Groups of 5 mice were pretreated with corn oil or with selected doses of TOTP and 18 hr later they were challenged with selected doses of malaoxon. The mice were sacrificed 5 min after challenge for brain cholinesterase determinations. The susceptibility of mice to malaoxon poisoning increased with increasing pretreatment doses of TOTP. Brain cholinesterase inhibition was used as the index of malaoxon toxicity. From the results (Fig. 1) the doses of malaoxon which would produce 50% inhibition of brain cholinesterase activity were estimated to be 27.0, 15.0, 7.0, 2.2 and 0.86 mg/kg in mice which had been pretreated with 0, 10, 17, 50 or 125 mg of TOTP/kg, respectively. The degree of potentiation of malaoxon toxicity was calculated by dividing the ID_{50} (malaoxon dose causing 50% inhibition of brain

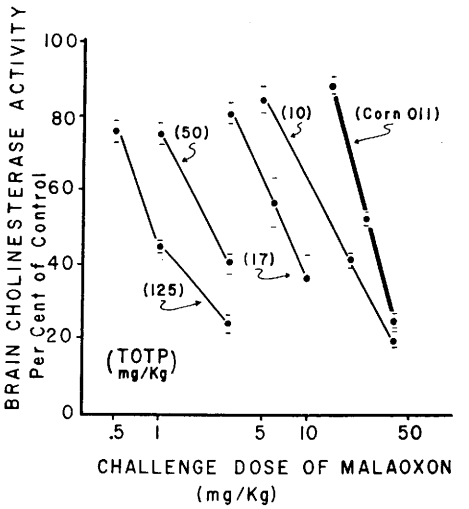

FIG. 1. Effect of TOTP pretreatment (18 hr) on malaoxon's anticholinesterase action, *in vivo*. Mice were sacrificed 5 min after malaoxon injection. Each point represents the mean (bracketed by the SE) of 5 mice.

cholinesterase) of malaoxon for corn oil pretreated mice by the ID_{50} of malaoxon in TOTP pretreated mice. Pretreatment with 10, 17, 50 and 125 mg/kg of TOTP resulted in 1.8-, 3.9-, 12.3- and 31.4-fold potentiation of malaoxon toxicity, respectively.

To compare malaoxon potentiation with carboxylesterase inhibition, groups of mice were sacrificed 18 hr after TOTP (without malaoxon challenge), the liver hydrolysis of diethyl succinate, triacetin and malathion and plasma hydrolysis of triacetin were determined. The results are shown in Fig. 2. Diethyl succinate esterase activity was more sensitive to inhibition by TOTP than the other esterases; however, all carboxylesterase activities studied were maximally inhibited by 50 mg of TOTP/kg. No significant increase in percentage inhibition of carboxylesterase activity was detected when the pretreatment dose of TOTP was increased from 50 to 125 mg/kg, although this dosage increment more than doubled the degree of potentiation of malaoxon toxicity. This suggested that an additional mechanism was involved in potentiation of malaoxon toxicity by high doses of TOTP.

One possible mechanism which might account for the observed increase in degree of

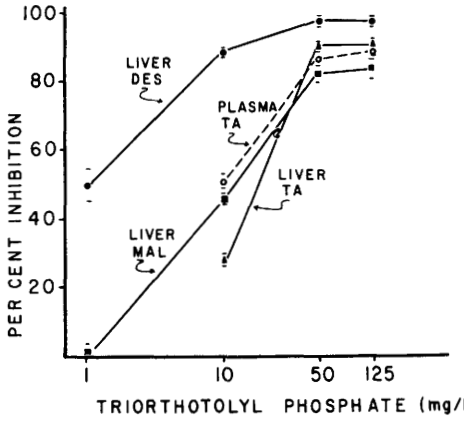

FIG. 2. Effect of TOTP on liver and plasma carboxylesterase activity. Mice were sacrificed 18 hr after TOTP for determination of liver and/or plasma hydrolysis of diethylsuccinate (DES), triacetin (TA) and malathion (MAL). Each point represents the mean (bracketed by the SE) of 5 mice.

potentiation is that the higher dose of TOTP might increase malaoxon toxicity by increasing the sensitivity of mouse brain cholinesterase to inhibition by malaoxon. To test this, groups of mice were pretreated with corn oil or with 10, 50 or 125 mg of TOTP/kg and were sacrificed 18 hr later for measurement of inhibition of brain cholinesterase by malaoxon *in vitro*. Malaoxon (0.25 nmoles) was preincubated with 50 mg of homogenized brain in Warburg flasks for 25 min. Acetylcholine was then added and cholinesterase activity was determined. Each brain tested served as its own control. The mean ± SE percentage inhibition of cholinesterase by 0.25 nmole malaoxon was 47.7 ± 1.1, 48.8 ± 0.9, 49.9 ± 0.9 and 50.7 ± 0.5 for brains from mice pretreated with 0, 10, 50, or 125 mg of TOTP/kg, respectively. Homogenized brain (50 mg) from one mouse of each treatment group was incubated with 0.5 or 1.0 nmole of malaoxon to compare sensitivity of cholinesterase to higher concentrations of inhibitor. All brains tested were inhibited 70 and 85% by 0.5 and 1.0 nmoles of malaoxon, respectively. Thus, TOTP had no detectable effect on the sensitivity of brain cholinesterase to inhibition by malaoxon.

Recently we demonstrated that mouse liver detoxified malaoxon by a noncatalytic binding-type mechanism which is inhibited by malathion-potentiating organophosphates (10). To determine if inhibition of malaoxon binding by TOTP might be related to the increased potentiation of malaoxon by the higher dose (125 mg/kg) of TOTP, groups of mice were pretreated with corn oil or with selected doses of TOTP, and were sacrificed without challenge 18 hr later for measurements of liver malaoxon binding activity. The mean ± SE percentage inhibition of malaoxon binding activity was 34.3 ± 6.4, 83.9 ± 0.9, 89.3 ± 2.9 and 98.6 ± 0.7 after 1, 5, 50 and 125 mg of TOTP/kg, respectively. The increase in inhibition of malaoxon binding from 89 to 99% when the TOTP dose was increased from 50 to 125 mg/kg was highly significant ($p < .004$), corresponding to the increase in degree of malaoxon potentiation following the increased TOTP pretreatment dose.

Since organophosphates inhibit pseudocholinesterase as well as carboxylesterase and acetylcholinesterase activities, malaoxon inhibition of pseudocholinesterase might serve as a mechanism of malaoxon inactivation. Any portion of an injected dose of malaoxon which binds to and inhibits the noncritical pseudocholinesterases would not be free to inhibit acetylcholinesterase, the more critical enzyme from the standpoint of toxic manifestations of malaoxon poisoning. If such were the case, prior inhibition of pseudocholinesterases by TOTP would decrease the effectiveness of the enzymes as a mechanism of malaoxon detoxification. To test this, groups of mice were pretreated with corn oil or with 10, 50, or 125 mg of TOTP/kg and were sacrificed 18 hr later for measurement of liver, lung, and plasma cholinesterase activity. For purposes of comparison brain cholinesterase activity was also determined for the same mice. The results are shown in Fig. 3.

Brain acetylcholinesterase activity was not significantly inhibited by any of the TOTP pretreatments. Liver, lung and plasma cholinesterase activities were 60 to 90% inhibited by 50 mg of TOTP/kg. As had been observed with liver and plasma carboxylesterase, plasma and lung cholinesterase activities were not further inhibited when the dose

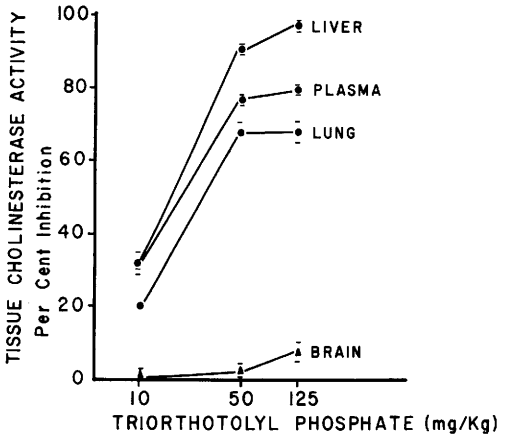

FIG. 3. Effect of TOTP on mouse tissue cholinesterase activity. Mice were sacrificed 18 hr after TOTP pretreatment. Each point represents the mean (bracketed by the SE) of 5 mice.

of TOTP was increased from 50 to 125 mg/kg. The increase in inhibition of liver cholinesterase when the pretreatment dose of TOTP was increased from 50 to 125 mg/kg was significant ($p < .05$). It appears therefore, that increased inhibition of liver cholinesterase was associated with the increased inhibition of liver malaoxon binding and with the increased degree of malaoxon potentiation.

Discussion. In this investigation we demonstrated that TOTP pretreatment increased the acute toxicity of subsequent doses of malaoxon in mice. In an attempt to elucidate the mechanism(s) responsible for the observed potentiation we studied the effect of TOTP on liver and plasma carboxylesterases, on the sensitivity of brain cholinesterase to malaoxon, *in vitro*, on liver binding of malaoxon and on liver, lung and plasma cholinesterases.

Carboxylesterase activities were maximally inhibited by 50 mg/kg of TOTP, yet malaoxon potentiation more than doubled when the TOTP pretreatment was increased to 125 mg/kg. This suggested that an additional mechanism, other than carboxylesterase inhibition, was responsible for the increase in potentiation after the higher dose of TOTP. Moreover, TOTP did not alter the sensitivity of brain cholinesterase to malaoxon *in vitro*.

Recent studies in our laboratories have demonstrated that, in addition to carboxylesterase hydrolysis, the *in vitro* inactivation of malaoxon by mouse liver included a noncatalytic, binding-type mechanism. Malaoxon binding by mouse liver was inhibited, *in vivo,* by malathion-potentiating doses of other organophosphates which also inhibited carboxylesterase activity (10). We and others have suggested that binding of potent cholinesterase inhibitors to noncritical tissue constituents can spare critical acetylcholinesterase and thus serve as a mechanism of protection against poisoning, and that inhibition of binding can increase the susceptibility of animals to poisoning by these compounds (10–13). In the present investigation liver binding of malaoxon, like liver diethyl succinate esterase activity, was significantly inhibited by as little as 1 mg of TOTP/kg, a dose which does not potentiate malathion or malaoxon toxicity. Malaoxon binding, unlike carboxylesterase activity, was not maximally inhibited by 50 mg of TOTP/kg. As the TOTP dose was increased from 50 to 125 mg/kg the malaoxon binding activity decreased from 11 to 1% of control activity. This decrease in binding corresponded to a more than twofold increase in degree of malaoxon potentiation following the increased TOTP dose, and suggests that inhibition of liver binding of malaoxon is responsible for malaoxon potentiation when carboxylesterase activity is maximally inhibited.

Brain acetylcholinesterase activity was not significantly inhibited by TOTP. Lung and plasma cholinesterase activities were maximally inhibited by 50 mg of TOTP/kg; whereas, liver cholinesterase, like liver malaoxon binding, was inhibited approximately 90% by this dose. Increasing the dose of TOTP to 125 mg/kg resulted in complete inhibition of the 10% liver cholinesterase activity and liver binding capacity which remained after 50 mg/kg. This suggests that pseudocholinesterases of the liver may contribute to malaoxon binding by mouse liver and that these enzymes may become most important as detoxification sites when carboxylesterases are completely inhibited.

Summary. The degree of potentiation of malaoxon toxicity by 10 to 50 mg/kg of

TOTP was associated with its inhibition of mouse liver carboxylesterase activity; however, the degree of potentiation increased with increasing dose of TOTP beyond the dose which caused maximal inhibition of liver carboxylesterase activity. TOTP did not alter the sensitivity of mouse brain cholinesterase to inhibition by malaoxon. As the pretreatment dose of TOTP was increased from 50 to 125 mg/kg there was an associated increase of inhibition of malaoxon binding by liver and increase in the inhibition of liver cholinesterase activity. These findings indicate that the increase in malaoxon potentiation beyond that detected at maximum inhibition of liver carboxylesterase activities by TOTP may result from increased inhibition of malaoxon binding. It appears that both carboxylesterases and cholinesterases may contribute to malaoxon binding by mouse liver, and that binding to liver cholinesterases may become the most important mechanism of inactivation of malaoxon when carboxylesterases are completely inhibited.

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