Inactivation of Malaoxon by Mouse Liver[1] (36368)

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Maloxon [*O,O*-dimethyl-*S*-(1,2-dicarbethoxyethyl) phosphorothiolate] is the active cholinesterase-inhibiting metabolite of the commonly used organophosphate insecticide malathion [*O,O*-dimethyl-*S*-(1,2-dicarbethoxyethyl) phosphorodithioate] (1, 2). The ethoxyethyl esters of both malathion and malaoxon have been shown to be hydrolyzed by carboxylesterase enzymes (E.C. 3.1.1.1) to form the corresponding monoacids which are not cholinesterase (CHE) inhibitors (3, 4). Malathion toxicity is potentiated by several other organophosphates, and this has been attributed to inhibition of carboxylesterases by the potentiating compounds (3–9).

Lauwerys and Murphy (10, 11) and Triolo, Mata, and Coon (12) obtained evidence that, in addition to enzyme catalyzed hydrolysis, binding of paraoxon (*O,O*-diethyl *O*-*p*-nitrophenyl phosphate) to nonvital tissue constituents is an important protective mechanism against paraoxon poisoning and that chemicals which inhibit or enhance this mechanism may increase or decrease paraoxon toxicity, respectively. The present investigation was undertaken to determine if a binding type of mechanism, similar to that suggested for paraoxon, is involved in the detoxification of malaoxon by mouse liver and in the potentiation of malaoxon anticholinesterase action by other organophosphates.

Materials and Methods. Male mice (Charles River, CD-1), weighing 20–30 g, were used. They were housed in air-conditioned rooms (75–80°F) and were supplied with food and water *ad libitum*. Parathion (*O,O* diethyl *O*-*p*-nitrophenyl phosphorothioate; 99%), TOTP (triortho tolyl phosphate; (practical grade), DEF (*S,S,S*-tribuytl phosphorotrithioate; 92%), EPN (*O*-ethyl *O*-*p*-nitrophenyl phenyl phosphorothioate; purified"), Abate [*O,O,O',O'*-tetramethyl *O,O'*-thiodi-*p*-phenylene phosphorothioate; 86%), ronnel [*O,O*-dimethyl *O*-(2,4,5-trichlorophenyl) phosphorothioate; 99%], Delnav [2,3-*p*-dioxanedithiol *S,S'*-bis(*O,O*-diethylphosphorodithioate): technical grade] and malaoxon (95%) were injected, ip, as corn oil solutions. The concentrations of the solutions were adjusted so that the volume injected was 5 ml/kg. Control animals were given corn oil only.

Mice were sacrificed by decapitation and exsanguination, and liver homogenates were prepared 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. The capacity of mouse liver to inactivate malaoxon was determined by a modification of the anticholinesterase (anti-CHE) method described by Lauwerys and Murphy (10). Except where indicated, 20 mμmoles of malaoxon were incubated with appropriate amounts of homogenized liver at either 1 or 38° in a 5.0-ml total volume of 0.02 *M* phosphate buffer, pH 7.6. At selected times the reaction was stopped by immersing the incubate in boiling water for 90 sec. The amount of malaoxon remaining was determined by a manometric (13) anticholinesterase assay in which 0.5-ml aliquots of the incubates were added to 0.39 μ*M* units of bovine erythrocyte cholinesterase (Sigma Chemical Co.) in a 3.0-ml calcium-free

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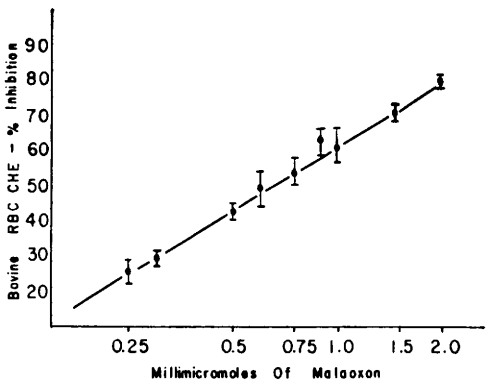

FIG. 1. Inhibition of bovine erythrocyte cholinesterase (CHE) by malaoxon *in vitro*: Each point represents the mean (bracketed by SE) of 4–6 separate assays.

Ringer–bicarbonate buffer system containing 0.01 *M* acetylcholine Cl as the substrate. The standard curve (Fig. 1) for inhibition of cholinesterase by malaoxon, prepared by incubating the enzyme with known amounts of malaoxon for 25 min before addition of substrate, was used to determine the malaoxon content of the aliquots taken from tubes containing liver and also from a malaoxon blank (no liver) which was carried through the same incubation and heating procedure. Malaoxon inactivation was calculated as the difference between the amount remaining in the liver incubate and in the blank (μmoles of malaoxon inactivated).

For carboxylesterase determinations, hydrolysis of 0.0067 *M* diethyl succinate by 2.5 mg and 0.027 *M* triacetin by 5.0 mg of homogenized liver was determined manometrically as described previously (14, 15).

Results. To compare liver hydrolysis of malaoxon with total liver inactivation of malaoxon, 5.3×10^{-3} *M* malaoxon was incubated for 25 min at 38° with 200 mg of liver in 3.0 ml of bicarbonate buffer in Warburg flasks; and the CO_2 production was measured. At the end of the incubation, an aliquot was removed from each flask, diluted with Ringer–bicarbonate buffer and immersed in boiling water to stop the reaction. The total amount of malaoxon inactivated was determined by the anti-CHE method. The amount of malaoxon hydrolyzed during the incubation was determined from the amount of CO_2 released from the buffer. The results of seven separate experiments indicated that 4240 ± 120 mμmoles (mean ± SE) of malaoxon were hydrolyzed in 25 min. In contrast, results of tests by the anti-CHE method indicated that 9120 ± 680 mμmoles of malaoxon were inactivated. The difference was highly significant by Student's *t* test ($p < .001$). This finding prompted a study of the time-course of malaoxon inactivation to further compare the two methods (manometric and anti-CHE) of measuring malaoxon inactivation *in vitro*. The results are shown in Fig. 2. In the manometric method, CO_2 production was determined at the indicated times after the addition of 5.3×10^{-3} *M* malaoxon to 200 mg of liver. For the anti-CHE method, 20 mμmoles of malaoxon were incubated with 20 mg of liver in phosphate buffer; and the reaction was stopped at the indicated times and the amount of malaoxon inactivated was determined. The results show that there was a nearly linear rate of production of CO_2 with respect to time between zero and 10 min. However more than 50% of the total 10-min malaoxon inactivation, as measured by the anti-CHE method, occurred during the first minute.

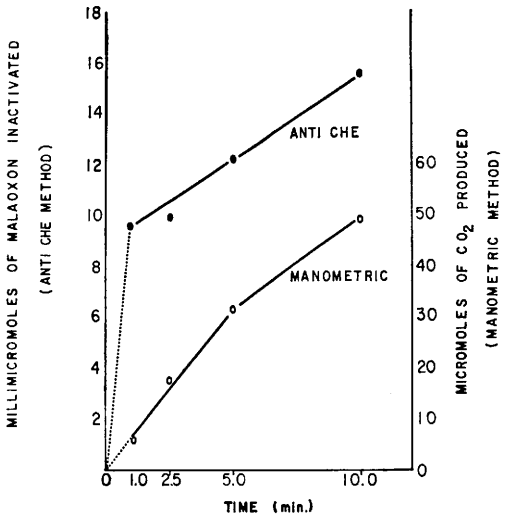

FIG. 2. Comparison of assay methods for malaoxon detoxification by mouse liver homogenates: Each point represents the average of at least 2 separate assays.

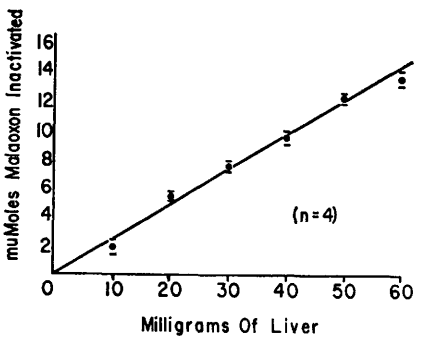

FIG. 3. Malaoxon inactivation by mouse liver during a 1-min incubation: Twenty μmoles of malaoxon were incubated with liver at 38°; and malaoxon inactivation was determined by the anti-CHE method. Each point represents the mean (bracketed by SE) of at least 4 separate assays.

In order to determine if the rapid inactivation of malaoxon was dependent upon the amount of tissue present, 20 mμmoles of malaoxon were incubated at 38° with 10 to 60 mg of liver for 1 min; and the amount of malaoxon inactivated was measured. The results (Fig. 3) indicate that malaoxon inactivation was linearly related to the amount of tissue present in the incubate.

Lauwerys (16) demonstrated that paraoxon binding by rat liver was independent of incubation temperature. If the rapid inactivation of malaoxon were due to a similar binding type of mechanism, it would also be expected to be independent of temperature. To test this, 20 mμmoles of malaoxon were incubated with 25 mg of liver at 1 and 38°; and the incubations were stopped at either 1 or 5 min. During the 5-min incubation period, 8.5 ± 0.3 mμmoles of malaoxon were inactivated at 1° and 13.7 ± 0.5 mμmoles at 38°. Thus, lowering the temperature reduced malaoxon inactivation by approximately 40%, ($n = 5$; $p < .01$). In contrast, during the 1-min incubation, 7.0 ± 0.2 and 7.1 ± 0.2 mμmoles of malaoxon were inactivated at 1 and 38°, respectively ($n = 5$, $p > .5$).

To determine the effect of longer incubations with mouse liver at 1°, 50 mg of liver were tested as this provided a total inactivation more easily measured by the anticholinesterase assay system. When 20 mμ moles of malaoxon were incubated with 50 mg of liver for 0.5, 1, 5, 10, and 20 min, 13.5 ± 0.2, 13.9 ± 0.3, 14.4 ± 0.2, 14.7 ± 0.2, and 15.7 ± 0.1 (mean ± SE; $n = 5$) mμmoles of malaoxon were inactivated, respectively. These data indicate that the rapid tissue-dependent inactivation of malaoxon at 1° was nearly complete within 30 sec. This is consistent with a binding mechanism of inactivation.

The effect of *in vivo* pretreatment of mice with other organophosphates on the binding of malaoxon by mouse liver *in vitro* was tested. Groups of 5 mice were pretreated with the compounds listed in Table I. Eighteen hours after injection the mice were sacrificed; and their livers were removed for measurement of malaoxon binding. Liver carboxylesterase activity was also determined with diethyl succinate and triacetin as substrates. At least one corn oil control was included with each test group, and control activities were pooled for comparison with those of the test animals. With the exception of parathion, all of the compounds tested have been shown to potentiate the acute toxicity of malathion and/or malaoxon (5, 8, 14, 17). All of the pretreatments, with the exception of the low dose of ronnel, caused significant ($p < .05$) inhibiton of both carboxylesterases and malaoxon binding.

Discussion. A comparison of the manometric and anti-CHE methods for measurement of malaoxon detoxification by mouse liver *in vitro* indicated that only about 50% of the total detoxification that occurred during a 25-min incubation at 38° was due to carboxylesterase hydrolysis of malaoxon. Lowering the incubation temperature from 38° and 1° had no detectable effect on the amount of malaoxon (7 mμmoles) inactivated during a 1-min incubation with 25 mg of liver; but it resulted in a decrease in the amount of malaoxon inactivated in 5 min from 13.8 to 8.5 mμmoles at 38 and 1°, respectively. The lack of a detectable effect of different incubation temperatures on the amount of malaoxon inactivated in 1 min is suggestive of a noncatalytic binding mechanism. If one assumes that approximately 7 mμmoles of malaoxon were inactivated by a noncatalytic mechanism at both 38 and 1°,

TABLE I. Effect of Organophosphates on Mouse Liver Carboxylesterases and Malaoxon "Binding."

Pretreatment[a]		Inhibition[b] (%)		
		Carboxylesterase		
Compound	Dose (mg/kg)	DES	TA	Malaoxon "binding"
Parathion	5	74 ± 2	75 ± 4	79 ± 3
TOTP	5	68 ± 2	22 ± 1	82 ± 1
DEF	20	22 ± 14	21 ± 11	26 ± 11
	50	96 ± 1	95 ± 1	99 ± 1
EPN	5	83 ± 4	—	79 ± 5
Abate	100	89 ± 3	82 ± 2	89 ± 3
Ronnel	25	10 ± 3	7 ± 7	4 ± 3
	50	47 ± 4	62 ± 3	56 ± 3
Delnav	10	68 ± 3	50 ± 3	66 ± 4

[a] Groups of 5 mice sacrificed 18 hr after ip injection.

[b] Activities of 8 control livers were 76.9 ± 7.2 and 49.9 ± 1.8 μl of CO_2/mg/15 min for diethyl succinate (DES) and triacetin (TA) esterase, respectively, and 13.0 ± 0.3 mμmoles of malaoxon inactivated/50 mg/1 min at 1° for malaoxon binding. Values are mean ± SE.

and subtracts this amount from 13.7 and 8.5 mμmoles, one is left with 6.7 and 1.5 mμmoles of malaoxon inactivated by a temperature-dependent mechanism at 38 and 1°, respectively. Thus, the malaoxon detoxification which was detected during the 5-min incubation period apparently consists of 2 components, one which is catalytic and one noncatalytic.

These data suggest that the anti-CHE method is more accurate than the manometric method in determining total malaoxon detoxification *in vitro* because it measures loss of the activity of malaoxon against its target enzyme. In contrast, the manometric method indirectly measures only the amount of malaoxon hydrolyzed, and will not detect any malaoxon detoxification which does not result in acid production and subsequent release of CO_2 from the bicarbonate buffer. In earlier studies, Murphy and DuBois (9) and Brodeur and DuBois (18) used the anti-CHE method for measuring malaoxon detoxification as an index of the capacity of tissues to detoxify malathion and as a correlation to measurements of the effects of various chemicals on malathion's toxicity. In view of the results of the present investigation which demonstrates that only about one-half the total detoxification of malaoxon can be accounted for by carboxylesterase activity, it seems probable that anti-CHE assays of malaoxon detoxification do not represent a true measure of the hydrolytic detoxification of malathion by carboxylesterases. In this study, the use of a shorter incubation period and lower temperature (1 min at 1°) for malaoxon inactivation studies was coupled with separate carboxylesterase assays and thus provided a convenient method for assessing the effect of other organophosphates on both components of malaoxon detoxification.

All of the organophosphates tested in this investigation inhibited malaoxon binding and carboxylesterase activity in mouse liver. It has been proposed that binding of the organophosphates, sarin (isopropyl methyl phosphorofluoridate) and paraoxon to noncritical tissue constituents can spare critical acetylcholinesterase and thus serve as a mechanism of protection against poisoning, and that inhibition of binding increases the susceptibility of animals to poisoning by these compounds (11, 19, 20). Results of this investigation suggest that a similar mechanism also exists for the detoxification of malaoxon in mouse liver. Table I supports

the suggestion (20) that carboxylesterase enzymes may be the noncritical binding sites for organophosphates since inhibition of malaoxon binding was closely associated with carboxylesterase inhibition (correlation coefficients = 0.9798, $p < .01$; and 0.7228, $p < .05$ for diethyl succinate and triacetin esterase activities, respectively). Thus malaoxon binding to noncritical tissue constituents may play a role in the potentiation of malaoxon and probably also of malathion by other organophosphates.

Summary. Comparison of the manometric and anti-CHE methods for measuring malaoxon detoxification *in vitro* demonstrated that carboxylesterase hydrolysis of malaoxon accounted for only about 1/2 of the total malaoxon detoxification detected by the anti-CHE method. Evidence was obtained which showed that there was a rapid, temperature-independent loss of anti-CHE activity of malaoxon in the presence of liver which suggested that the discrepancy was due to noncatalytic binding of malaoxon to tissue constituents. Malaoxon binding was inhibited by malathion-potentiating doses of other organophosphates which alsc inhibited carboxylesterase activity, *in vivo*. A close dose–effect correlation was observed between carboxylesterase inhibition and inhibition of malaoxon binding. This suggests that carboxylesterase enzymes may act as noncritical binding sites for malaoxon inactivation. Malaoxon binding, as well as catalytic hydrolysis, may play a role in malaoxon detoxification and inhibiton of binding may contribute to potentiation of malaoxon by compounds which compete for binding sites.

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