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MALATHION A AND B ESTERASES OF MOUSE LIVER—I

SEPARATION AND PROPERTIES*

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Abstract—The hydrolysis of malathion [S-(1,2-dicarbethoxyethyl) *O,O*-dimethyl phosphorodithioate] is catalysed by two types of enzymes in the mouse liver. The first is malathion B-esterase which hydrolyses one of the carboxyethyl ester groups giving malathion mono-acid. This is a B type esterase, being sensitive to low concentrations of DFP (diisopropyl phosphorofluoridate), paraoxon (diethyl *p*-nitrophenyl phosphate) and EPNO the oxygen analogue of EPN (*O*-ethyl *O*-*p*-nitrophenyl phenylphosphorothionate). It is predominently in the microsomes, acts at pH 7·4–7·6 and is probably the same as the non-specific carboxylesterases (EC 3.1.1.1). The second enzyme is malathion A-esterase which is insensitive to DFP and other organophosphates even at high concentrations. It is predominently in the cell-sap, has an optimum pH of 8·8, requires 2-mercaptoethanol, reduced glutathione or similar SH compounds for activation, and is inhibited by *p*-chloromercuribenzoate and heavy metals. The A esterase degrades malathion at the P–S linkage giving *O,O*-dimethyl phosphorothioate.

The molecule of malathion [S-(1,2-dicarbethoxyethyl) *O,O*-dimethyl phosphorodithioate] can be enzymatically broken at least six points (Fig. 1) [1]. By administering ^{32}P-labelled malathion to mice, hens and cockroaches several metabolites have been isolated [2–4]. These have been broadly classified as products due to carboxylesterase and phosphatase activities [3], but knowledge about the enzymes which catalyse these reactions is inadequate. In mammals, the detoxification of malathion is effected mainly by the carboxylesterase pathway [1]. The first attempt at identification of malathion hydrolysing enzymes in the mammalian system was made by Cook *et al.* [5] who showed that degradation occurred at one of the carboxyethyl ester groups. The enzyme which was called malathionase was shown to be sensitive to organophosphorus esters like paraoxon and EPNO (parathion and EPN treated with bromine water). The chief degradation product was malathion monoacid [6] now known to be malathion α-monoacid [7]. The work of Main and Braid [8] with a partially purified mouse liver preparation shows that malathionase is probably the same as the non-specific carboxylesterases (EC 3.1.1.1) which belong to the B type [9].

There is even less information on the "phosphatase" type of enzymes which by inference [2–4] act on malathion at points other than the carboxyethyl ester groups. In the present study it is shown that in addition to the B type of esterase which acts at the carboxyethyl ester group, there is an A type of esterase in mouse liver which breaks the linkage at P–S (Fig. 1). Whereas most of the previous workers [5, 6, 8] have followed malathion esterase activity by the acid liberated by a manometric or pH-stat procedure, we have employed a colorimetric procedure recently developed by us [10] which determines the rate of disappearance of the carboxyl ester function of malathion.

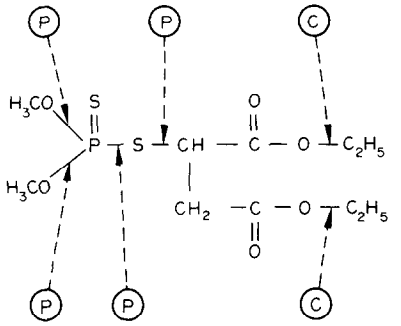

Fig. 1. Structural formula of malathion with possible points of enzymic degradation. C = carboxylesterases; P = phosphatases (O'Brien [1]).

MATERIALS AND METHODS

Malathion used in these studies was a secondary standard of 98·9% purity supplied by American Cyanamid Co. Agricultural Division, Princeton, N.J. An 0·1 M stock solution was prepared in methanol and kept in the refrigerator. Appropriate volumes were blown into 0·05 M Tris–HCl buffer (pH 7·6 for B esterase and 8·8 for A esterase) containing 0·1% (w/v) Triton X-100 to obtain uniform suspensions. Malaoxon, malathion mono- and di-acids and *O,O*-dimethyl phosphorodithioate were also obtained from the same source. EPN (*O*-ethyl *O*-*p*-nitrophenyl phenylphosphorothionate) which was of analytical grade was obtained from E. I. du Pont de Nemours and Co., Wilmington, Delaware. For preparation of EPNO, the oxygen analogue, the method of Neal and DuBois [11] was used, the EPNO content being

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assayed by the method of Bhagwat and Ramachandran [12]. DFP (diisopropyl phosphorofluoridate) and paraoxon (*O,O*-diethyl *O*-*p*-nitrophenyl phosphate) were supplied by Sigma Chemical Co. Appropriate quantities of a 10% stock solution were weighed and diluted with buffer just before use. Cysteine, reduced glutathione (GSH) and dithiothreitol were also Sigma products. 2-Mercaptoethanol was obtained from Fluka AG, Switzerland and Sephadex from Pharmacia, Uppsala. Other chemicals were of analytical or chemically pure grade obtained through local sources.

Male and female albino mice (25–40 g) of a Swiss strain maintained in our colony were used in all experiments. The animals were killed by stunning and decapitation. The livers were removed quickly and homogenized with the appropriate buffer, or 0·25 M sucrose when subcellular components were required, in a Potter-Elvehjem type of homogenizer rotating at 1000 rev/min. All operations were at 0–4°. The homogenizing medium also contained 10^{-3} M 2-mercaptoethanol when malathion A-esterase activity was to be determined.

Malathion B-esterase was determined as described previously [10] with minor modifications. The digest contained 10^{-3} M malathion, 0·5% liver, 0·05 M Tris-HCl buffer pH 7·6 with 0·1% Triton X-100. Aliquots of 5 ml were withdrawn at intervals of 5 min up to 30 min, extracted with an equal volume of cyclohexane, and the residual malathion in the extract was determined after evaporation at 110°–120°. After development of the ferric-hydroxamate colour, a small amount of a filter aid (Hyflo-Supercel, ICI) was added and the solution filtered before determination of colour. For malathion A-esterase activity the system contained 0·05 M Tris-HCl buffer pH 8·8 containing 0·1% Triton X-100 and 10^{-3} M 2-mercaptoethanol, 10^{-3} M GSH, 10^{-5} M DFP and 2% liver. The liver homogenate was incubated with the necessary amount of DFP and GSH for 30 min before addition to malathion substrate. The rest of the procedure was as for malathion B esterase.

RESULTS

Malathion B-esterase. The present experiments were restricted to establish optimum conditions of assay of this activity by the newly developed ferric-hydroxamate method [10]. The malathion in the digest was found to decrease linearly with time up to 40 min. In general the activity was followed for 30 min. The activity was proportional to the concentration of liver in the digest in the region of 0·2 to 0·6% wet weight. In general a concentration of 0·5% was used. The solubility of malathion in water is low, being 145 mg/l. Some kind of dispersing agent is thus necessary. Triton X-100 at 0·1% was found suitable. Main and Braid [8] have given the K_m value of malathion for carboxylesterase as $0{\cdot}057 \pm 0{\cdot}013 \times 10^{-3}$ M. We have tried three concentrations, viz., 0·5, 1·0 and $1{\cdot}5 \times 10^{-3}$ M. The malathion B-esterase activities were 91, 86 and 81 μmoles/g liver/hr, respectively. The progressive slight decrease in activity as the substrate concentration is increased is explained by the possible presence in the malathion sample used by us of malaoxon or isomalathion [13] which are both inhibitors of B-esterases. We therefore used a constant substrate concentration of 1×10^{-3} M malathion in all experiments.

Table 1. Malathion B-esterase activity in the presence of various concentrations of organophosphates

Concn of inhibitor (M)	Per cent activity of control			
	DFP	Paraoxon	EPNO	Malaoxon
None	100	100	100	100
10^{-9}	90			
10^{-8}	75	77	93	
10^{-7}	67	60	56	
10^{-6}	23	34	34	
10^{-5}	0–14	0–13		93
10^{-4}	0–12	0–12		50
10^{-3}	0–12	0–12		

Mouse liver homogenate was incubated for 30 min at 0° with the indicated concentrations of the inhibitors before addition to 10^{-3} M malathion suspension in 0·05 M Tris–HCl buffer, pH 7·6. Malathion B-esterase activity was determined at 37° as described under Methods. EPN + EPNO mixtures were prepared from EPN by oxidation with bromine-water according to Neal and DuBois [11] and the EPNO content determined and adjusted according to Bhagwat and Ramachandran [12]. The values at inhibitor concentrations of 10^{-5} M and above were not consistent. Higher enzyme concentrations were used at inhibitor concentrations of 10^{-6} M and above.

Malathion B-esterase activity was found to be 120 ± 17 (S.D.) μmoles/g of liver per hr ($n = 25$). There was no sex difference, the values in one set of experiments being 109 for females and 112 for males ($n = 5$; S.E.M. $\pm$ 4·5; $P > 0{\cdot}25$ by the Student's *t*-test). Since malathion hydrolysing activity in a crude preparation like liver homogenate may be due to more than one enzyme, it is not possible to determine the accurate values of pI_{50} for various organophosphates by conventional methods [14, 15]. The activities after incubation with different concentrations of DFP, paraoxon, EPNO and malaoxon are given in Table 1. It is seen that the pI_{50} is in the range 6–7 for the first three organophosphates. The incubations were invariably for 30 min at 0°. The concentrations needed to effect 50% inhibition were much lower (10^{-9} to 10^{-8} M) in the case of microsomes and much higher (about 10^{-6} M) in the case of cell-sap. Malaoxon inhibited B-esterases by 50% at 10^{-4} M when homogenates were used The values at inhibitor concentrations above 10^{-4} M could not be determined as the ferric-hydroxamate colour due to malaoxon itself interfered in the determinations.

Malathion A-esterase. It is seen from Table 1 that malathion B-esterase activity in liver homogenates is not completely inhibited by DFP and paraoxon which are known to be powerful inhibitors of B-esterase activity. The values for residual activity were erratic and ranged from 0 to 14 μmoles of malathion hydrolysed/g liver per hr. It was suspected that the residual activity might be due to a type of esterase which is insensitive to organophosphates (A-esterases). Since A-esterases are usually SH enzymes and sometimes have an optimum pH different from that of B-esterases, several preliminary experiments were tried to determine the optimum conditions for assay.

Table 2. The activating effect of some SH compounds on malathion A esterase activity

Compound added	Malathion A-esterase activity (μmoles/g liver per hr)	Control (%)
None	2·5	100
Cysteine	4·2	168
Dithiothreitol	12·0	480
2-Mercaptoethanol	15·2	608
Glutathione (reduced)	25·0	1000

8 ml of a 10% mouse liver homogenate in 0·05 M Tris-HCl buffer pH 8·8 were incubated with 10^{-5} M DFP and 10^{-3} M activator (2 ml) for 30 min at 0°. This was added to 30 ml of malathion suspension in the same buffer containing 40 μmoles of malathion. Malathion A esterase activity was determined as described under Methods.

To determine the activation by SH reagents; the homogenate was treated with 10^{-5} M DFP (paraoxon at the same concentration gave similar results) to suppress all B-esterase activity. This was done by incubation for 30 min at 0°. To this homogenate the appropriate activators were added at 10^{-3} M concentration, and after 30 min the malathion esterase activity was determined. The activating effect of a few SH reagents is given in Table 2. The results show that malathion hydrolysing activity is considerably increased by the addition of SH containing substances. GSH has the highest activating effect when all the reagents were at 10^{-3} M concentration. By increasing the concentration or mixing any two activators the effect was not further improved. For routine assays the buffer contained 10^{-3} M 2-mercaptoethanol. Incubation of the homogenates was done simultaneously with 10^{-5} M DFP and 10^{-3} M GSH.

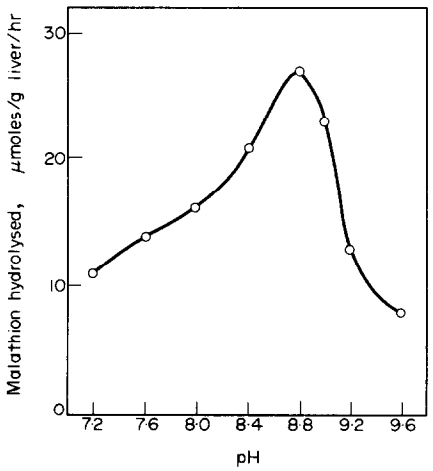

Fig. 2. pH–activity relationship of malathion A-esterase of mouse liver. The assay system contained 0·05 M buffer (Tris–HCl up to pH 9·0 and glycine–NaOH from pH 9·2 to 9·6), 0·1% Triton X-100, 10^{-3} M 2-mercaptoethanol and 10^{-3} M malathion. The homogenates was preincubated with 10^{-5} M DFP and 10^{-3} M GSH for 30 min before addition to the substrate. Values for pH 9·2 and above have been corrected for non-enzymic hydrolysis. Other details are given in the text.

Employing the conditions given in the previous section, malathion A-esterase activity was determined at various pH values from 7·2 to 9·6. The buffers used were 0·05 M Tris–HCl up to pH 9·0 and glycine–NaOH from pH 9·2 to 9·6. The pH–activity curve is given in Fig. 2. It is seen that the optimum pH is fairly sharp at 8·8. There was no non-enzymatic hydrolysis of malathion up to pH 9·0. Corrections have been made in the graph for the slight hydrolysis at pH 9·2 and above.

Stability of malathion A and B esterases. Homogenates preserved under toluene retained the B-esterase activity for at least 48 hr. On the other hand, the A-esterase activity was almost lost in 3 hr at 0° if no activator such as 2-mercaptoethanol was added. In the presence of 10^{-3} M 2-mercaptoethanol the activity was undiminished for 24 hr at 0°, and for at least 3 days (maximum period tried) in a frozen condition. The malathion A-esterase activity in mouse liver is about 25 μmoles/g of liver per hr in the presence of 10^{-3} M GSH. This represents about 20% of the B-esterase activity.

Subcellular distribution of A and B-esterases. Mouse liver was homogenized in 0·25 M sucrose and the subcellular fractions were isolated by conventional methods [16], the microsomes being separated by centrifugation at 105,000 *g* for 1 hr. The pellets were suspended in buffer at pH 7·6 or 8·8 depending whether B or A esterase activity was to be determined. Table 3 gives the distribution of the activities. In the case of A esterase the enzymes were preincubated as usual with DFP and GSH and the buffer contained 10^{-3} M 2-mercaptoethanol. The major part of the B-esterase activity was found in the microsomal fraction. This is consistent with the findings of several authors that the non-specific carboxylesterases are predominently microsomal enzymes [17–20]. The B-esterase activity in the "nuclear" and mitochondrial fractions may be enhanced due to microsomal contamination.

Effect of metals and inhibitors on malathion A-esterase. SH enzymes are known to be sensitive to metallic ions. Since malathion A-esterase is inactive except in the presence of GSH and other SH activators and

Table 3. Subcellular distribution of malathion A and B esterase activities

Fraction	Per cent of total activity	
	A esterase	B esterase
Nuclei and cell debris	13·7	14·6
Mitochondria	7·8	21·6
Microsomes	10·8	47·5
Cell sap	67·7	16·4

The subcellular fractions were prepared from mouse liver by the method of Hogeboom [16] in a Spinco Model L ultracentrifuge using 0·25 M sucrose as medium. The pellets were suspended in 0·05 M Tris–HCl buffer, pH 7·6 for B esterase and pH 8·8 buffer containing 10^{-3} M mercaptoethanol for A esterase. The fractions were incubated with 10^{-5} M DFP and 10^{-3} M GSH for 30 min at 0° for A esterase determination. The values are averages of 5 runs for B esterase and 3 runs for A esterase. The procedure for the determination of malathion A and B esterase is given under Methods.

since these activators themselves form complexes with metallic salts it is difficult to study their activity and determine the concentrations needed to effect a 50% inhibition. The data presented are therefore qualitative. For these studies, the supernatant fraction after dialysis against 0·01 M Tris–HCl buffer pH 8·8 containing 0·001 M 2-mercaptoethanol for 6 hr (two changes of medium) or after passing through Sephadex G-25 with the same medium as effluent was employed. Malathion A-esterase is inhibited partially by *p*-chloromercuribenzoate at 10^{-3} M and completely at 5×10^{-3} M. Iodoacetate at 10^{-3} M completely inhibits the activity. The following metals also completely inhibit the activity at 10^{-3} M; Hg^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Ba^{2+}. A esterases are known to be activated by certain metals but the sensitivities differ with different enzymes [21, 22]. It is known that Mn^{2+} activates DFPase [21] in the kidney and the soluble fraction of the liver tissue, while it inhibits the DFPase of the particulate fractions. Ca^{2+} activates paraoxon hydrolysing enzymes [22]. To determine whether malathion A-esterase belongs to any of these classes, the effect of Mn^{2+} and Ca^{2+} was thoroughly investigated. The effect of Ca^{2+} was negligible. Mn^{2+} inhibited the homogenate enzyme by 45% at 10^{-3} M. The inhibition was much lower (16%) with cell-sap. If malathion esterase is identical with DFPase and consists of two components, the insoluble component inhibited by Mn^{2+} and the soluble part activated, then the difference in the sensitivities of the whole homogenate and the cell-sap can be explained. However, the malathion A-esterase activity in the particulate fractions was so small (Table 3) that no conclusive results could be obtained.

Metabolic pathways of A and B esterases. Malathion was enzymatically hydrolysed by the A and B malathion esterases as described. The unreacted malathion was removed by extraction with cyclohexane, the aqueous phase was acidified to pH 2·0 with sulphuric acid and heated in a boiling water-bath to coagulate the proteins. After removal of the precipitate the filtrate was extracted with ether to obtain malathion mono-acid and other organic acids if present. The aqueous phase was concentrated *in vacuo*. Paper chromatography by the method of Plapp and Casida [23] showed that malathion monoacid was the only metabolite of B esterase activity. This was absent when A esterase acted on malathion. In this case the only metabolite which could be identified was *O,O*-dimethyl phosphorothioate. There was no trace of the dithioate as tested by the copper complex method of Orloski [24]. To test whether the dithioate was first formed and then enzymatically degraded, the compound was incubated with liver homogenate for 1 hr as for A esterase determination. The dithioate was quantitatively recoverable from the enzymic digest as the copper complex. These results show that malathion A-esterase acts mainly at the P–S bond and not at the S–C bond (see Fig. 1).

Attempts to isolate the other metabolite which should be diethyl thiomalate (2-mercaptosuccinic diethyl ester) were unsuccessful. This ester as well as diethyl succinate, diethyl fumarate and diethyl maleate are extractable to the extent of about 70% from aqueous suspensions by an equal volume of cyclohexane. When these cyclohexane extracts were shaken with alkaline hydroxylamine as described in the methods for the separation of malaoxon from malathion [25] or EPNO from EPN [12] the esters were easily leached into the aqueous phase where they could be detected by the addition of hydrochloric acid and ferric chloride. Malathion is not leached into the hydroxylamine phase under these conditions. When tested by this method no thiomalic ester was detectable in the enzymic digests. During the evaporation of the cyclohexane to obtain the residual malathion these esters were found to be completely lost. Thus, if any thiomalic ester was present it would not be found in the final malathion residue, and would not interfere in the malathion A-esterase determination.

To see if thiomalic ester first formed might have been further degraded by enzymic activity, the ester was treated with liver homogenate as for malathion A-esterase activity, and the rate of disappearance was determined by extracting the residual ester with cyclohexane and leaching it with alkaline hydroxylamine. It was found that thiomalic diethyl ester was rapidly degraded by malathion A-esterase. No thiomalic acid or malic acid could be detected by paper chromatography in enzymic digests of malathion with the A esterases. Either these are further metabolized or the quantities are too small for detection by paper chromatography.

DISCUSSION

In the present work it is shown that in addition to the already known malathion esterase, which is a B-type esterase there is another enzyme belonging to the A-type which acts on malathion. This enzyme is insensitive to DFP and paraoxon. Whereas the B-esterase is predominantly in the microsomes, has a pH optimum in the region of 7·4 to 7·6 and hydrolyses malathion at the carboxyethyl ester group, the A esterase is predominantly in the cell-sap, has a pH optimum of 8·8 and hydrolyses malathion at the P–S bond. The A-esterase is almost inactive as such but is activated by cysteine, 2-mercaptoethanol and reduced glutathione. The term malathion A-esterase is used loosely since strictly speaking an esterase of the A type should not only not be inhibited by an organophosphate but should hydrolyse it. Pure malathion is not an inhibitor of B esterase but malaoxon is. The name malaoxonase may be more appropriate, since in another communication it will be shown that only the A type hydrolyses malaoxon. However, the term malathion A-esterase is used in this paper to distinguish it from malathion B-esterase.

The A-type of esterases which hydrolyse organophosphorus esters are known to belong to a heterogenous class [21, 22, 26]. Kojima and O'Brien [22] found that in the mouse liver there were three types of paraoxon degrading enzymes with different pH optima. It is likely that the malathion A esterase described in this paper may thus consist of several components.

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