Hydrolysis of Malathion by Ali-Esterases *in vitro* and *in vivo*

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The structure of malathion, *S*-[1,2-di(ethoxy-carbonyl)ethyl] *OO*-dimethyl phosphorodithioate, has suggested to many investigators (e.g. Murphy & Dubois, 1957; Seume & O'Brien, 1960) that hydrolases may be involved in the synergism observed by Frawley, Fuyat, Hagan, Blake & Fitzhugh (1957) between malathion and *O*-ethyl *O*-*p*-nitrophenyl phenyl phosphorothioate. On the basis of their known substrate specificities, ali-esterases (carboxylic ester hydrolase, EC 3.1.1.1), cholinesterases and lipases might be expected to contribute to the enzymic hydrolysis of the ethyl esters of malathion. By the same criteria (Main, 1960), serum A-esterases were not considered as likely possibilities. The A-esterases of other tissues were not investigated.

Ali-esterases (Myers & Mendel, 1953), also known as 'non-specific esterases' and 'liver esterases', are B-type esterases as defined by Aldridge (1953). The terms 'non-specific esterases' and 'liver esterases' are misleading, since ali-esterases exhibit a definite if broad specificity pattern and occur in many tissues besides the liver. They are probably identical with the B-esterases of the liver and serum and, as will be shown, hydrolyse both aromatic and aliphatic uncharged carboxylic esters. Unlike cholinesterases, they readily hydrolyse simple aliphatic esters such as ethyl butyrate and do not hydrolyse acetylcholine and similar substrates.

Serum and liver cholinesterases, like ali-esterases, readily hydrolyse glycerol triesters, such as tributyrin (Strelitz, 1944), and aromatic esters such as phenyl butyrate and *o*-nitrophenyl butyrate (Main, Miles & Braid, 1961). Lipases appear to work best on emulsified substrates (Aldridge, 1954), whereas ali-esterases hydrolyse substrates in the aqueous phase. Both readily hydrolyse simple aliphatic esters.

Cook, Blake & Yip (1958) and Yip & Cook (1959) have observed enzymic hydrolysis of the ethyl esters of malathion by rat-liver homogenates and by extracts of rat-liver acetone-dried powders. However, the authors used crude preparations and did not establish the number and identity of the esterases contributing to their 'malathionase' activity, nor did they place the hydrolysis on a quantitative basis. Yip & Cook (1959) and, later, Murphy, Anderson & Dubois (1959) have speculated

that the inhibition of 'malathionase' might be involved in the synergisms observed between malathion and such compounds as *O*-ethyl *O*-*p*-nitrophenyl phenyl phosphorothioate, parathion and tri-*o*-tolyl phosphate, but it was not always clear what type of hydrolase the authors had in mind when they referred to 'malathionase'.

In the present work the contribution of ali-esterases, cholinesterases and lipases to the hydrolysis of malathion was investigated. Since considerable overlapping of substrate-specificity patterns occurs between these classes of esterases it seemed desirable to examine the action of each esterase on malathion with preparations essentially free from other esterases. Differentiation in crude tissue preparations was then placed on a more secure basis. For this purpose horse serum cholinesterase and liver ali-esterase from rat and human were partially purified. In addition, a commercial preparation of pancreatic lipase was tested. On the basis of the results obtained *in vitro*, the hydrolysis of malathion under physiological conditions was investigated.

EXPERIMENTAL

Materials

Abbreviation. EPN, *O*-ethyl *O*-*p*-nitrophenyl phenyl phosphorothioate.

Malathion. Three grades of malathion were available. All were prepared by the American Cyanamid Co., New York 20, N.Y., U.S.A. The malathion used for work *in vitro* was labelled '99.6% pure'. A stock solution of malathion was prepared by diluting 0.166 g. of 99.6% malathion to 10 ml. with methanol. Substrate solutions were prepared by blowing 1 ml. of this into about 90 ml. of water containing 5 ml. of 2% acacia and diluting to 100 ml.

The malathion used to dose animals was of two grades: one was labelled 'secondary standard, purity 96.8%'; the other was labelled 'malathion, technical, purity 95.6%'.

Parathion. Parathion (*OO*-diethyl *O*-*p*-nitrophenyl phosphorothioate) and paraoxon, (diethyl *p*-nitrophenyl phosphate) were purified research-grade samples supplied by Albright and Wilson Co.

Tri-*o*-tolyl phosphate. The 'practical grade' was purchased from Eastman Organic Chemicals.

Lipase. Pig pancreas, crude, was purchased from Mann Res. Labs. Inc., New York, U.S.A.

Rat liver and serum. Male rats of the Sprague-Dawley strain were used as a source of tissues and for dosing experiments. Typical control and test groups contained 10 rats.

each weighing 200–300 g. The blood and livers were taken immediately after stunning the rats. Fibrin was removed by stirring the blood with a glass rod. The blood was then centrifuged and the serum was removed by siphon. The erythrocytes, if required, were washed three times with an iso-osmotic NaCl solution and were then dialysed against weakly buffered water at pH 7. The livers were cooled immediately after removal and were then homogenized with an equal volume of water in a blender. Both serum and liver homogenates were stored under toluene in the cold.

Human livers. Two human livers, one from a girl aged 17 years and the other from a woman aged 70 years, were obtained within 2 hr. of accidental death. The livers were stored frozen and when required were homogenized in the same manner as described above.

Oral dosing. Tri-*o*-tolyl phosphate was given undiluted to either unstarved rats or starved rats, depending on the interval between the tri-*o*-tolyl phosphate and malathion doses. When the interval was 1 hr., the rats had been starved for about 24 hr. when they received the former. When the interval was 24 hr., the tri-*o*-tolyl phosphate was given to fed rats which were then starved before receiving the malathion.

Secondary standard malathion was given undiluted or diluted in propylene glycol (25 mg./ml.) to rats that had previously been starved for about 24 hr. Technical malathion was diluted in corn oil and given in two concentrations: 500 and 50 mg./ml. Preliminary experiments indicated no significant difference between the use of corn oil and propylene glycol as diluent under the conditions of these experiments.

METHODS

Colorimetric measurement of ali-esterase. Routine determinations of rat serum and liver activities were made with *o*-nitrophenyl butyrate (Main *et al.* 1961) as substrate at 25° and at pH 6.3. The cholinesterase activities of rat serum and liver, although significant at higher pH values, at pH 6.3 were negligible. The ali-esterase activity at pH 6.3 was, however, near its maximum, making the method sensitive and specific for ali-esterase. Usually 0.02 ml. of serum or 1 mg. of liver, suitably homogenized and diluted, was required for each determination. The *o*-nitrophenol was measured at 371 m μ .

Measurement of activities. Cholinesterase and ali-esterase activities were also determined with a radiometer pH-stat. The acid liberated by ester hydrolysis was titrated at a pre-set, constantly maintained pH with standard NaOH.

In most determinations 20 mM-NaOH, delivered from a 0.5 ml. syringe, was used, making a total available titre of 10 μ moles. When substrate concentrations were below 0.1 mM, 2 mM-NaOH was used as titrating agent. At these concentrations absorption of atmospheric CO₂ interfered with the titration. To overcome this difficulty mechanical stirring was replaced by a stream of N₂ bubbled through the reaction medium. The volume of standardized NaOH required was automatically plotted against time on a Titrigraph recorder. The slope over the initial 5 min. was taken as the reaction rate. With esters of limited solubility, stock solutions in methanol ranging from 0.5 to 2M were prepared. Appropriate volumes of these (1 ml. or less) were then blown into the required aqueous media and made to a

final volume of 100 ml. Suitable controls with the inhibitors, di-isopropyl phosphorofluoridate or eserine, were run with every enzymic determination to correct the total hydrolysis rate and obtain the hydrolysis rate for the particular esterase being studied. It was necessary to control the temperature of the reaction medium to within $\pm 0.1^\circ$.

Manometric method. The methods used have been described by Aldridge (1953) and Main *et al.* (1961). The acetylcholine concentration was 6 mM when brain and erythrocyte cholinesterases were determined and 30 mM when serum cholinesterases were determined.

Determination of protein. Routine measurement of serum protein was made by determining the light-absorption at 280 m μ with a silica u.v. spectrophotometer. The protein in liver preparations was determined by the biuret method when possible (Layne, 1957). Otherwise, the micro-Kjeldahl method of Chibnall, Rees & Williams (1943) was used. Both the biuret and spectrophotometric method at 280 m μ were calibrated against the Kjeldahl method. Kjeldahl nitrogen was converted into protein on the assumption that protein contained 16% of nitrogen.

RESULTS

Ali-esterases. Rat-liver preparations purified 33- and 55-fold with respect to initial protein were employed to determine the action of ali-esterase on malathion. These preparations were intermediate products from the purification method. The following description is included to characterize the partially purified ali-esterase products employed.

Cholinesterases were not present as judged by the absence of activity towards acetylcholine and of inhibition by eserine with *o*-nitrophenyl butyrate (Main *et al.* 1961). A-type esterases were judged to be absent since di-isopropyl phosphorofluoridate inhibited all the activity towards *p*-nitrophenyl acetate, which is readily hydrolysed by A-type esterases in the presence of the phosphorofluoridate (Aldridge, 1953). The phosphorofluoridate inhibited all the activity toward *o*-nitrophenyl butyrate, *p*-nitrophenyl acetate, ethyl acetate, propionate, butyrate and valerate, as well as a number of other esters, which suggested that ali-esterase was responsible for these activities. Summation experiments with mixed substrates and partial inactivation by heat also suggested the presence in the preparation of only one esterase hydrolysing *o*-nitrophenyl butyrate and ethyl butyrate. It was concluded that these preparations were free from esterases other than ali-esterase.

Human ali-esterase was partially purified by the same method, but the purification was not as great (about sixfold). However, this preparation also appeared to be free from other esterases by the criteria used for the rat-liver ali-esterase.

The substrate-specificity pattern of the esterase in the rat- and human-liver preparations is shown in Table 1. The pattern of activities with respect to

Table 1. Comparison of the substrate-specificity patterns of partially purified rat- and human-liver ali-esterases and of rat serum ali-esterase

Activities were related to ethyl butyrate (100 for each preparation). Rat liver was purified 55-fold and human liver sixfold. Rat-serum-ali-esterase activity was determined in the presence of $10\text{ }\mu\text{M}$ - eserine. The activities in terms of μmoles of ethyl butyrate hydrolysed/mg. of protein/min. were: 55-fold rat liver, 161; sixfold human liver, 14.8; rat serum, 0.0694. Activities were determined with a pH-stat at pH 7.6 and 37° . Substrate concentrations (mM) are given in parentheses.

Substrate	Rat liver (55-fold)	Human liver (sixfold)	Rat serum
Ethyl butyrate (20)	100	100	100
Ethyl acetate (20)	8	2.3	3.8
Ethyl propionate (20)	33	24	58
Ethyl valerate (20)	55	54	84
Phenyl butyrate (10)	288	85	575
<i>o</i> -Nitrophenyl butyrate (1.5)	252	360	168
Malathion (0.5)	7.6	1.8	89

Table 2. K_m and $V_{\max}$ values of various tissue preparations with malathion

Activities were determined in duplicate with 0.5, 0.2, 0.1 and 0.05 mM -malathion at 37° , pH 7.6 (by pH-stat). Standard errors were calculated by the method of Wilkinson (1961).

Preparation	$V_{\max}$		K_m (μM)
	$\mu\text{moles/g. of}$ protein/min.	$\mu\text{moles/ml. or}$ g. wet wt./min.	
Human-liver homogenate (aged 17)	41 ± 2	7.91 ± 0.33	48 ± 10
Human-liver homogenate (aged 70)	$29.8^* \pm 0.5$	$5.7^* \pm 0.1$	—
Human serum	Nil	Nil	—
Human erythrocytes	Nil	Nil	—
Rat-liver homogenate	44.5 ± 2.8	9.55 ± 0.58	57 ± 13
Rat serum	84.8 ± 3.0	5.67 ± 0.20	56 ± 7.1
Purified human-liver ali-esterase (sixfold)	242 ± 15	—	83 ± 11
Purified rat-liver ali-esterase (33-fold)	1360 ± 97	—	166 ± 27

* Activity at 0.5 mM -malathion only. $V_{\max}$, assuming K_m is $45\text{ }\mu\text{M}$, would be $32.5\text{ }\mu\text{moles/g. of protein/min.}$

the simple aliphatic esters was similar. However, the similarity was less close when aromatic esters were considered.

The comparable specificity pattern for rat serum ali-esterase is also given in Table 1. Activities were measured in the presence of $10\text{ }\mu\text{M}$ - eserine to eliminate possible cholinesterase activity.

Both partially purified liver ali-esterase preparations and rat serum ali-esterase hydrolysed malathion, as shown in Table 1. Serum ali-esterase exhibited a much higher ratio of malathion to ethyl butyrate activity than did the liver ali-esterases, emphasizing further that serum and liver ali-esterases differ significantly. Subsequently one of us (P.E.B.) has shown that rat serum ali-esterase is quickly and irreversibly destroyed by butan-1-ol under conditions that leave liver ali-esterase relatively unaffected. It may therefore be concluded that purified liver ali-esterase is essentially free from the small amount of serum ali-esterase that would normally be expected to contaminate it, since high concentrations of butan-1-ol are used in the purification procedure.

The kinetic properties of the hydrolysis of malathion by ali-esterases were investigated. The ali-esterase activities of the purified liver preparations,

of crude liver homogenates and of rat serum were measured at various malathion concentrations. K_m and $V_{\max}$ values were calculated by the method of Wilkinson (1961) (Table 2). The K_m values of rat-liver homogenate and serum and of human-liver homogenate were similar and low, suggesting a close affinity between malathion and the ali-esterases. The activity of human liver was comparable with that of rat liver, but human blood was devoid of enzymic activity toward malathion.

The products from the ali-esterase hydrolysis of malathion did not inhibit ali-esterases or serum cholinesterases.

The effect of pH on the activity was determined. The activity reached a plateau at about pH 6.9, which extended to pH 9, when it appeared to decrease slightly. The optimum activity range covered the physiological pH range.

Extent of hydrolysis of malathion by ali-esterases. Table 3 gives the result of an experiment that was performed to determine whether one or both of the ethyl esters of malathion are acted on by ali-esterase. Purified (55-fold) rat-liver ali-esterase was employed. Since $5.0\text{ }\mu\text{moles}$ of malathion were added, complete hydrolysis of both ethyl esters would require the addition of $10\text{ }\mu\text{moles}$ of NaOH.

The reaction had practically stopped when 4.95 μ -moles of alkali had been added (Table 3). The addition of fresh enzyme did not result in further hydrolysis. It was concluded that ali-esterase hydrolysed only one ethyl ester from malathion. Similar experiments with rat serum and human-liver-ali-esterase preparations gave the same results. These results are in agreement with the observation of Cook & Yip (1958) that 'malathionase' yields a monoacidic malathion acid. It is not certain which of the two acids possible is produced or whether both are produced.

Cholinesterases. Strelitz (1944) stage 2 purified (42-fold) horse-serum-cholinesterase preparation was tested against malathion and a number of other substrates. The results are given in Table 4. Ethyl butyrate and tributyrin are included to give some indication of the point at which the substrate specificities of ali-esterases and cholinesterases cease to overlap. They both hydrolyse one type of aliphatic ester (the glycerol esters), but only ali-esterases hydrolyse simple aliphatic esters of the ethyl butyrate type at significant rates. This distinction may help in understanding the further observation that the cholinesterases did not hydrolyse malathion at a significant rate (less than 1/500 the rate of acetylcholine hydrolysis).

Preincubation of malathion (0.5 mM) with cholinesterases and mixed (malathion and acetylcholine) substrates gave essentially the same

activity as with acetylcholine alone, suggesting that cholinesterases have no affinity for malathion and would not significantly modify its toxicity.

Pancreatic lipases. An acetone-dried powder of pig pancreatic lipase was investigated for malathion activity. An aqueous 5% (w/v) suspension in 0.1M-NaCl was prepared and the pH adjusted to 7.6. The activity of this material towards a 100 mM-tributyrin emulsion was 820 μ moles/g. of dry powder/min. at 37°, pH 7.6 as measured on the pH-stat. A 1 mM-malathion suspension was hydrolysed slowly (0.71 μ mole/g. of dry powder/min.), but the activity toward an aqueous 0.5 mM solution was negligible. Under physiological conditions it therefore appeared unlikely that lipases would significantly modify malathion toxicity.

Effect of a practical grade of tri-o-tolyl phosphate on various esterases in rat serum and liver. Myers & Mendel (1953) have shown that tri-o-tolyl phosphate is a powerful inhibitor of rat-liver ali-esterase *in vivo*. A practical grade of tri-o-tolyl phosphate (0.5 g./kg.) was therefore given orally to rats and the results are shown in Fig. 1. The serum cholinesterase and brain and erythrocyte acetylcholinesterases were not inhibited as rapidly or as completely as the ali-esterases. The serum ali-esterases were reduced to nil in 60 min. and the liver esterases to 12% of the initial activity. The liver ali-esterase was reduced further after 24 hr. to 1.5% of the initial activity.

Table 3. *Extent of the hydrolysis of the ethyl ester groups of malathion by purified rat-liver ali-esterase*

To 5.00 μ moles of malathion in 10 ml. of water was added 0.133 ml. of 55-fold-purified rat-liver ali-esterase. The rate and extent of hydrolysis was measured with the pH-stat at pH 7.6 and at 37°.

Time from addition of ali-esterase (min.)	Hydrolysis rate (μ moles/ml./min.)	Percentage of initial hydrolysis rate	Malathion hydrolysed (μ moles)	Percentage to the completion of hydrolysis of one ethyl ester
0	6.9	100	0	0
5	2.7	54	3.60	72
10	0.16	2.3	4.75	95
35	0.011	0.16	4.95	99

Table 4. *Serum-cholinesterase hydrolysis of malathion and other substrates*

Activities were measured by pH-stat at pH 7.6 (25°) with 0.2 ml. of human serum or 0.1 ml. of 42-fold-purified horse-serum-cholinesterase preparation (Strelitz, 1944, stage 2). Eserine (0.02 mM) controls accompanied every determination. Substrate concentrations (mM) are given in parentheses.

Source of cholinesterase	Substrate(s)	Initial velocity (μ moles/ml./min.)	Relative activities (acetylcholine = 1)
Horse (purified)	Acetylcholine (30)	12.4 $\pm$ 0.4	1.0
	Acetylcholine (30), malathion (0.5)	12.2 $\pm$ 0.2	0.99
	Malathion (0.5)	0.017 $\pm$ 0.008	0.0014
	Tributyrin (30)	3.90 $\pm$ 0.10	0.31
	Ethyl butyrate (20)	0.05 $\pm$ 0.01	0.004
Human	Acetylcholine (30)	2.94 $\pm$ 0.2	1.0
	Malathion (0.5)	<0.005	0
	Ethyl butyrate (20)	<0.005	0

Table 5. Rate of inhibition of rat serum ali-esterase in vivo by malathion

Secondary standard malathion was given orally. Blood samples were taken from the tails. Ali-esterase was determined colorimetrically with *o*-nitrophenyl butyrate at pH 6.3 and at 25°.

Malathion (mg./kg.)	Initial activity (μ moles/ml. of serum/min.)	Percentage of initial activity remaining after indicated interval between dosing and sampling					
		0.5 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
1250	5.80	76.5	35.3	12.0	—	6.5	9.8
1250	0.79	Rat died shortly after dose					
1500	13.70	48	39.2	31.5	30.3	28.5	—
1500	11.18	69	56.7	37.8	35.5	36.8	35.0
1700	3.06	25.5	0	0	Died after 2 hr.		
1700	12.67	73.2	48.2	31.2	25.8	21.3	—

Inhibition of serum ali-esterase in vivo by malathion. Serum-ali-esterase activities were determined before administration of a single oral dose of malathion and at intervals after dosing. The results are given in Table 5. The initial ali-esterase activity of one rat was unusually low and this rat died within minutes of dosing (our Sprague-Dawley colony was destroyed by an epi-zootic that began just as this experiment was performed; the unusually low ali-esterase activity noted may be due to this). In any case, it is evident that malathion inhibits ali-esterase *in vivo*. The activity dropped rapidly in the first hour after dosing and then tended to level off. The degree of inhibition appears to be a function of the initial activity as well as of the dosing level.

*Toxicological significance of the inhibition of cholinesterases after dosing with tri-*o*-tolyl phosphate.* Cholinesterases were significantly inhibited after dosing with tri-*o*-tolyl phosphate (Fig. 1). It was therefore of interest to determine whether the compound contributed significantly to the inhibition of acetylcholinesterase leading to death. This possibility was tested with parathion and paraoxon, whose toxicities are not significantly modified by ali-esterase.

If the toxicity of parathion or paraoxon was significantly increased by pre-dosing with tri-*o*-tolyl phosphate, it could be assumed that the last-named was responsible for the increase, and that it would also increase the apparent malathion toxicity.

Pre-dosing starved male rats with 0.5 g. of tri-*o*-tolyl phosphate/kg., followed in 24 hr. by parathion and paraoxon, did not significantly decrease the LD₅₀ of either compound (Table 6). Indeed the tri-*o*-tolyl phosphate increased the parathion LD₅₀ by a factor of 2, which suggested that it inhibited the rate of conversion of parathion into paraoxon. This explanation seems probable, since pre-dosing with tri-*o*-tolyl phosphate had no significant effect on the paraoxon toxicity. It was concluded that the inhibition of cholinesterases by tri-*o*-tolyl phosphate was of no toxicological significance.

Fig. 1. Effect of a single oral dose (0.5 g./kg.) of tri-*o*-tolyl phosphate on brain (A) and erythrocyte (C) acetylcholinesterase, serum cholinesterase (B) and liver (D) and serum (E) ali-esterases of the rat as a function of time. Cholinesterase activities were determined manometrically with acetylcholine and the ali-esterases colorimetrically with *o*-nitrophenyl butyrate. Initial activities were: serum cholinesterase, 14.2 μ l. of CO₂/ml./min.; erythrocyte acetylcholinesterase, 2.59 μ l. of CO₂/g. of protein/min.; brain acetylcholinesterase, 41.2 μ l. of CO₂/g. of protein/min.; serum ali-esterase, 2.39 μ moles/ml./min.; liver ali-esterase, 256 μ moles/g. of protein/min.

*Effect on toxicity of malathion of inhibition of ali-esterase with tri-*o*-tolyl phosphate.* The acute oral LD₅₀ of malathion was determined with control rats and with rats that previously had been given an oral dose of a practical grade of tri-*o*-tolyl phosphate (0.5 g./kg.) to inhibit the ali-esterase activity (Fig. 1).

The results of dosing with secondary standard malathion are given in Table 7. The LD₅₀ was 1600 mg./kg. When the interval between dosing with tri-*o*-tolyl phosphate and malathion was 1 hr. the LD₅₀ was 35 mg./kg. When the interval was increased to 24 hr., the LD₅₀ was reduced to 20 mg./kg. or by a factor of 80.

The LD₅₀ of technical malathion was 415 mg./kg. Dosing with tri-*o*-tolyl phosphate, followed in 24 hr. by technical malathion, reduced the LD₅₀ to 7.5 mg./kg. or by a factor of 53 (Table 6).

Table 6. *Effect, on the acute oral LD₅₀ of technical malathion, parathion and paraoxon, of pre-dosing rats with 0.5 g. of a practical grade of tri-o-tolyl phosphate/kg.*

Male rats (220 ± 40 g.) were starved for 24 hr. on screens before dosing with malathion, parathion and paraoxon. Tri-o-tolyl phosphate was given to unstarved rats 24 hr. before LD₅₀ dosing. Typically three or four groups consisting of ten rats each were used to establish each LD₅₀. Appropriate dosing ranges were established with preliminary experiments. LD₅₀ values were estimated graphically. The interval between dosing and death of the group closest to the LD₅₀ is given.

Compound	Treatment	LD ₅₀ (mg./kg.)	Interval between dosing and death (hr.)
Parathion	Control, no tri-o-tolyl phosphate	4.3	0.6 $\pm$ 0.1
	Tri-o-tolyl phosphate	8.3	0.9 $\pm$ 0.2
Paraoxon	Control, no tri-o-tolyl phosphate	2.8	0.5 $\pm$ 0.1
	Tri-o-tolyl phosphate	2.6	0.5 $\pm$ 0.1
Technical malathion	Control, no tri-o-tolyl phosphate	415	3.5 $\pm$ 0.8
	Tri-o-tolyl phosphate	7.5	2.0 $\pm$ 0.6

Table 7. *Effect on the acute oral LD₅₀ of secondary standard malathion, of orally pre-dosing with a practical grade of tri-o-tolyl phosphate*

Male rats (200 ± 50 g.) were starved for 24 hr. on screens before administration of malathion.

Malathion (mg./kg.)	Mortality	Mortality (%)	Time to death (hr.)
1000	0/12	0	—
1250	1/10	10	12
1500	5/12	42	12
1750	8/10	80	One at 8*
2000	9/12	75	One at 6*

LD₅₀ 1600 mg./kg.

Dose: 0.5 g. of tri-o-tolyl phosphate/kg., followed in 1 hr. by malathion

12.5	0/10	0	—
20	0/10	0	—
27.5	1/9	11	6
35	5/10	50	2.4 $\pm$ 1.9
42.5	8/10	80	2.1 $\pm$ 1.3
60	7/7	100	0.3 $\pm$ 0.13†

LD₅₀ 35 mg./kg.

Dose: 0.5 g. of tri-o-tolyl phosphate/kg., followed in 24 hr. by malathion

10	2/10	20	2.4 $\pm$ 1.3
15	5/10	50	1.0 $\pm$ 0.7
20	5/10	50	0.9 $\pm$ 0.6
25.0	14/20	70	0.5 $\pm$ 0.25
32.5	12/20	60	0.6 $\pm$ 0.5
40	9/10	90	0.6 $\pm$ 0.5

LD₅₀ 20 mg./kg.

* Remaining rats died overnight between 12 and 24 hr. after dosing, except for one rat that died 25 hr. after dosing.

† One rat died overnight.

The acute oral LD₅₀ of secondary standard malathion was 3.7 times as high as that of technical malathion, which suggested that technical malathion might contain esterase-inhibiting impurities from which the standard grades of malathion were known to be free. This possibility was investigated by incubation of various concentrations of technical

malathion with rat serum for 60 min. at 37°. After incubation ali-esterase was determined colorimetrically, cholinesterase manometrically. Technical malathion inhibited both esterases. The plot of inhibition pI gave pI_{50} 2.96 for ali-esterase and pI_{50} 2.25 for cholinesterase. These results suggested that the impurities in technical malathion inhibited ali-esterase more readily (by a factor of 5) than cholinesterase.

DISCUSSION

Of the esterases examined, only the ali-esterase hydrolysed malathion at significant rates. The products of hydrolysis did not inhibit ali-esterase and cholinesterase and it seems improbable that they would be converted, *in vivo*, into inhibiting compounds. This hydrolysis, *in vivo*, would therefore be a detoxicating one, destroying malathion before it is converted into malaaxon.

The effect of this detoxicating activity on the acute toxicity of malathion was examined *in vivo* after treatment with tri-o-tolyl phosphate, which inhibited serum- and liver-ali-esterase activity, as shown in Fig. 1. The acute toxicity of malathion, expressed as the oral LD₅₀, is compared in Table 8 with the inhibition of ali-esterase activity and with the ali-esterase activity expressed in terms of the enzymic half-life ($t_{0.5}$), (see below), at comparable intervals after administration of tri-o-tolyl phosphate. The close correspondence between the activity and the LD₅₀ suggested that ali-esterase was a dominant factor influencing malathion toxicity in rats.

This conclusion is supported by calculating the approximate time necessary for the rat ali-esterases to hydrolyse a normal LD₅₀ dose of malathion at saturation concentration and then to reduce it to a sublethal concentration.

When the malathion concentration is 0.5 mM (saturation), the serum ali-esterase activity alone will hydrolyse an oral LD₅₀ dose in about 17 min.

The time necessary to reduce the malathion concentration from 0.5 to 0.001 mM (assuming 0.001 mM to be sublethal) was calculated by substituting typical K_m and V values into the integrated Michaelis equation (Dixon & Webb, 1958)

$$\left(\frac{2.303}{t}\right) \log \left(\frac{s_0}{s_0 - y}\right) = \left(\frac{V}{K_m}\right) - \left(\frac{1}{K_m} \cdot \frac{y}{t}\right)$$

where s_0 is the initial substrate concentration and the concentration at time t is $(s_0 - y)$. When V is 5 μ moles of malathion/g. of tissue/min. (37°, pH 7.6) and K_m is 50 μ M, the time required is 72 min. An LD₅₀ dose of malathion, when it kills, typically does so in several hours. It is evident therefore that the initial ali-esterase activity is more than sufficient to hydrolyse an LD₅₀ dose of malathion and reduce the concentration to sublethal levels under toxicological conditions. This calculation therefore supports the conclusion suggested by the results *in vivo* that ali-esterases are a dominant factor determining the acute toxicity of malathion.

Inhibition of ali-esterase after administration of malathion was rapid within the first hour and then tended to level off (Table 5). This more immediate inhibition may explain to some extent why the malathion LD₅₀ is lower than the initial ali-esterase activity would suggest.

The enzymic half-life is given by the expression $(t_{0.5})_e = 0.695 (K_m/V)$, where $(t_{0.5})_e$ is the time necessary to reduce a given substrate concentration at constant enzyme concentration to half its initial value. It is independent of the substrate concentration and may be derived as a special case of the integrated Michaelis equation.

(V/K_m) is not a true first-order rate constant since it has the dimensions min.⁻¹ [enzyme]⁻¹. Consequently the enzymic half-life is dependent on

the enzyme concentration and is not a real but a limiting value, which is approached only as (K_m/s) approaches infinity.

Expression of activity as $(t_{0.5})_e$ was considered to be more useful than expression in the more usual form because, for toxicological purposes, it is desirable to include a measure of the affinity of the enzyme for its substrate (K_m) as well as the rate of reaction (V), since substrate concentrations *in vivo* are typically both very low and variable.

The LD₅₀ in the complete absence of ali-esterase is difficult to assess precisely. When the serum-ali-esterase activity had been reduced to nil with tri-*o*-tolyl phosphate, and the liver activity, expressed as $(t_{0.5})_e$, was about 250 min., the LD₅₀ was 20 mg./kg. with secondary standard malathion. With technical malathion under the same conditions it was 7.5 mg./kg. Technical malathion contained impurities that preferentially inhibited ali-esterase. If the impurities in the LD₅₀ dose of 7.5 mg./kg. inhibited the residual ali-esterase activity under these conditions, then the LD₅₀ of malathion in the absence of ali-esterase, but after a dose of tri-*o*-tolyl phosphate, is about 7.5 mg./kg.

Tri-*o*-tolyl phosphate increased the LD₅₀ of parathion by a factor of 2. If the compound had the same effect on malathion conversion, then the true malathion LD₅₀ in the complete absence of ali-esterase would be about 4 mg./kg. The sublethal concentration of malathion previously mentioned was based in part on this LD₅₀ calculation.

Seume & O'Brien (1960) have shown that malathion is metabolized by rat tissues to yield, predominantly, malathion mono- and di-acid, which suggested the action of a carboxyl esterase. EPN inhibited this hydrolysis, as well as inhibiting the oxidation of malathion to malaonoxon. Frawley *et al.* (1957) observed that EPN potentiated the toxicity

Table 8. Comparison of the acute oral LD₅₀ of malathion with the ali-esterase activity, expressed as the enzymic half-life $(t_{0.5})_e$ of malathion and as percentage inhibition of the normal activity

Inhibition and activity values were calculated from the effect of tri-*o*-tolyl phosphate on ali-esterase measured *in vivo* (Fig. 2) and from inhibition *in vitro* of ali-esterase by technical malathion. (S), Serum; (L), liver. Human-liver malathion $(t_{0.5})_e$ was 4.2 min.; that of serum was infinity.

Grade of malathion	Interval between tri- <i>o</i> -tolyl phosphate and malathion	Interval between malathion dosing and death* (hr.)	LD ₅₀	Percentage inhibition of normal ali-esterase	Activity as $(t_{0.5})_e$ (min.)
Secondary Standard	No tri- <i>o</i> -tolyl phosphate	18†	1600	0	6.8 (S) 4.2 (L)
Secondary Standard	1 hr.	2.4 ± 1.9	35	100 (S) 88 (L)	∞ (S) 50 (L)
Secondary Standard	24 hr.	0.9 ± 0.6	20	100 (S) 98.5 (L)	∞ (S) 395 (L)
Technical	No tri- <i>o</i> -tolyl phosphate	3.5 ± 0.8	415	66 (S and L)	15 (S and L)
Technical	24 hr.	2.0 ± 0.6	7.5	100 >98.5	∞†

* Interval with experimental dose closest to LD₅₀ value calculated.

† Estimated.

of malathion. The EPN-induced inhibition of carboxyl ester hydrolysis of malathion together with its potentiation of malathion toxicity is in agreement with the conclusion suggested in the present work: that ali-esterase hydrolysis *in vivo* is a dominant factor influencing malathion toxicity.

Murphy & Dubois (1957) have shown that EPN inhibited detoxication of malaaxon by rat tissues. The detoxicating enzyme was more sensitive to inhibition by EPN *in vivo* than were the cholinesterases. Malaaxonases capable of hydrolysing the phosphorus ester bond would in all probability not be inhibited by organophosphorus compounds such as EPN. These results therefore suggest that ali-esterases are capable of hydrolysing the carboxyl esters of malaaxon as well as of malathion. However, as Table 5 shows, malathion, *in vivo*, as malaaxon, inhibits ali-esterase. This evidence suggests tentatively therefore that malaaxon is hydrolysed by and inhibits ali-esterase. If this is the case, ali-esterase has a double detoxicating action, acting on both malathion and malaaxon. The present kinetic evidence would suggest that hydrolysis of malathion is the predominant detoxicating reaction of ali-esterase, but the hydrolysis of malaaxon may be of considerable significance.

The production of malathion dicarboxylic acids, although not attributable to ali-esterase, which hydrolyses malathion only to the mono-acid, may be regarded as a further step in malathion degradation. But this further degradation is not necessarily a detoxicating step since the mono-acid derivative is already non-toxic.

In the present work, it was observed that tri-*o*-tolyl phosphate decreased the toxicity of parathion. Tri-*o*-tolyl phosphate had no effect on paraoxon toxicity, which suggested that it inhibited oxidation of parathion to paraoxon. The inhibition of malathion conversion into malaaxon by EPN reported by Seume & O'Brien (1960) may well influence the toxicity of malathion in the same way that tri-*o*-tolyl phosphate influenced parathion toxicity. Tri-*o*-tolyl phosphate and EPN may therefore influence malathion toxicity in two oppositely acting ways. The first is to inhibit ali-esterase and thereby increase the toxicity. The second is to inhibit oxidation to malaaxon and thereby decrease the toxicity.

EPN, unlike tri-*o*-tolyl phosphate, has a powerful 'anticholinesterase' toxicity of its own. The oral LD₅₀ of EPN for rats is 36 mg./kg. (Gaines, 1960). The final toxicity of combinations of EPN and malathion would therefore be partly due to the toxicity of EPN itself, in addition to the two factors mentioned above.

The hydrolysis of malathion by ali-esterases explains the vast difference between the toxicity of compounds such as parathion and malathion. The

susceptibility of ali-esterases to inhibition by organophosphorus compounds explains in large part the synergisms observed between malathion and tri-*o*-tolyl phosphate, EPN and similar compounds.

It is difficult at this time to predict precisely the toxicity of malathion toward human beings on the basis of the detoxicating effect of ali-esterases in the rat. The complete absence of ali-esterase activity in human serum means that at least one important barrier to malathion poisoning present in rats is absent in humans.

SUMMARY

1. Partially purified rat- and human-liver ali-esterases free from other esterases hydrolysed malathion, yielding 1 equivalent of acid for every mole of malathion present.

2. The activity in human- and rat-liver homogenates and in rat serum attributable to ali-esterases hydrolysed malathion at rates and under conditions which suggested that the ali-esterases would significantly modify malathion toxicity. Human blood contained no enzyme capable of hydrolysing malathion.

3. The malathion K_m and V_{max} values were determined for the various ali-esterases.

4. The serum- and liver-ali-esterases activities of rats were lowered to almost negligible levels by an orally administered dose of tri-*o*-tolyl phosphate. The cholinesterases were not inhibited to the same degree and for toxicological purposes did not appear to be inhibited to a significant degree.

5. The LD₅₀ of malathion was lowered by factors of the order of 100 by the previous administration of tri-*o*-tolyl phosphate.

6. It was concluded that ali-esterases were a dominant factor governing the toxicity of malathion and that their inhibition was largely responsible for the synergisms observed between malathion and other organophosphorus compounds.

7. Serum cholinesterases did not hydrolyse malathion at a significant rate, nor did pancreatic lipase.

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Tricarboxylic Acid Cycle and Related Enzymes in Cell-Free Extracts of *Mycobacterium tuberculosis* H₃₇R_v

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It has been fairly well established that several bacteria possess the tricarboxylic acid cycle for the terminal oxidation of carbohydrates. Youmans & Youmans (1953) could not demonstrate the growth of *Mycobacterium tuberculosis* H₃₇R_v from small inocula in the presence of the intermediates of the tricarboxylic acid cycle. They suggested that this might be due rather to the impermeability of the bacterial cells to these compounds than to the fact that the cells lacked the corresponding enzymes. In support of this theory, Youmans, Millman & Youmans (1956) presented qualitative data on the oxidation of all the intermediates of the tricarboxylic acid cycle by acetone-dried cells of the organism. However, no correlation was made between the amount of substrate disappearing and of oxygen consumed, nor were the oxidation products identified. Indira & Ramakrishnan (1959) have already shown that *M. tuberculosis* utilizes both the glycolytic and pentose phosphate pathways for the catabolism of glucose to the stage of pyruvic acid. The present study was undertaken to detect the individual enzymes of the tricarboxylic acid cycle in cell-free extracts of *M. tuberculosis* H₃₇R_v.

MATERIALS AND METHODS

Chemicals. Citric acid, α -oxoglutaric acid, fumaric acid, sodium hydrogen malate, 2,6-dichlorophenol-indophenol and triphenyltetrazolium chloride were obtained from British Drug Houses Ltd.; NADP⁺, NADH, NADPH, CoA, horse-heart cytochrome c, phenazine methosulphate,

glyoxylic acid (sodium salt), thiamine, pyrophosphate, acetyl phosphate and FAD were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; oxaloacetic acid, GSH, DL-isocitric acid lactone and lipoic acid were from California Corp. for Biochemical Research, Calif., U.S.A.; cis-aconitic acid, DL-isocitric acid (trisodium salt) and FMN were from Nutritional Biochemicals Corp., Ohio, U.S.A.; NAD⁺, ATP and ADP were from Pabst Laboratories, Milwaukee, Wis., U.S.A.; sodium succinate, sodium acetate and sodium pyruvate were from E. Merck A.-G., Darmstadt, Germany; vitamin K₃ (menadione), vitamin K₁ and α -tocopherol acetate were from Hofmann-La Roche and Co. Ltd., Basle, Switzerland; bovine serum albumin fraction V was from Armour and Co., Chicago, Ill., U.S.A.; [2-¹⁴C]acetate was from The Radiochemical Centre, Amersham, Bucks. Acetyl-CoA was prepared according to the method of Stadtman (1957).

Test organism. *Mycobacterium tuberculosis* H₃₇R_v, originally obtained from the National Collection of Type Cultures, London, was maintained on Petrik medium (Gradwohl, 1948). It was cultured in the liquid synthetic medium (Youmans & Karlson, 1947).

Whole-cell suspensions. The procedure used was essentially that of Youmans *et al.* (1956). After 14 days of incubation at 37°, the cells were harvested by filtration, washed with water, dried between several folds of filter paper and ground in a mortar with phosphate buffer (0.05M, pH 7.0) to make a homogeneous suspension containing 100 mg. of cell material/ml.

Cell-free extracts. Whole-cell suspensions prepared as described above, except that the pH of the suspending buffer was 6.0, were subjected to oscillation in a 10 kcy. Raytheon sonic oscillator for 20 min. at 3–6°. The preparation was centrifuged at 10 000g for 60 min. at 0–5° in a Servall high-speed centrifuge and the supernatant was used as the source of the crude enzyme. Alternatively, cells were