Studies on Ali-esterases

5. SUBSTRATE SPECIFICITY OF THE ESTERASES OF SOME SAPROPHYTIC MYCOBACTERIA*

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Recent investigations by Mendel, Myers, Uyldert, Ruys & de Bruyn (1953) and by Ruys (1954) have shown that the growth of *Mycobacterium tuberculosis* can be inhibited by relatively small amounts of various organophosphorus compounds under specified conditions. A similar finding had been briefly reported for *Lactobacillus arabinosus* (C. Haber, quoted by Brooks, Ransmeier & Gerard, 1949). However, Mendel *et al.* (1953) further pointed out that the concentrations of the organophosphorus compounds required to inhibit the growth of the mycobacteria corresponded closely with the concentrations which inhibit the ali-esterases of these bacilli.

Further investigations showed that the growth of certain strains of saprophytic mycobacteria was also very sensitive to inhibition by the organophosphorus compounds (Ruys, unpublished results), whereas the growth of other strains was scarcely affected. This observation offered an opportunity to investigate the suggested correlation between the effects of these inhibitors on the growth and on the ali-esterases of the mycobacteria. In the course of these investigations, it became apparent that some of the mycobacteria must contain more than one ali-esterase. The substrate specificities and other characteristics of these enzymes were therefore studied with the aid of selective inhibitors as described in previous investigations (cf. Myers, 1956).

EXPERIMENTAL

Substrates and inhibitors. The substrates included the esters used in previous investigations (Myers, Schotte, Boer & Borsje-Bakker, 1955; Myers, 1956) together with ethyl phenylacetate (Wislicenus, 1897), acetanilide and butyranilide (Gerhardt, 1853).

The inhibitors used were diethyl *p*-nitrophenyl phosphate (E600, paraoxon), diisopropyl *p*-nitrophenyl phosphate (DINP), and phenyl *N*-*n*-propylcarbamate. E600 and DINP were obtained through the courtesy of Albright and Wilson Ltd., London. Phenyl *N*-*n*-propylcarbamate (m.p. 53–54° after recrystallization from *n*-hexane) was synthesized from phenol and *n*-propyl isocyanate by the

general method of Kobezen, Metcalf & Fuhuto (1954); the isocyanate derivative was prepared by the procedure described by Schroeter (1909).

Determination of esterase activity. Esterase activity was measured by a manometric method in the Warburg apparatus at 37.5° and pH 7.4 in a medium containing 0.025 M-NaHCO₃ and saturated with CO₂ + N₂ (5:95, v/v). Preliminary experiments at 25° and 37.5° indicated that the latter temperature was suitable for measuring the esterase activities of the saprophytic mycobacteria as well as those of mammalian tissues. The amount of enzyme preparation was usually adjusted so that the activity was in the range of 2–8 µl. of CO₂/min. in each experiment. The reaction mixture was incubated for 30 min. at 37.5° with or without inhibitor before adding a small volume of substrate, in order to determine the residual esterase activity. All esters were prepared in the form of an aqueous emulsion, suspension or solution containing 5% (w/v) of gum acacia; the final concentrations of ester and gum acacia in the reaction mixture were 0.2 and 0.1% (w/v) respectively. The esterase activity was calculated from the initial constant rate of hydrolysis of the substrate after the appropriate corrections had been made for slow spontaneous hydrolysis.

The rate of hydrolysis of butyranilide and acetanilide was also measured by the manometric technique described above (cf. Michel, Bernheim & Bernheim, 1937); in this case, the concentration of substrate was increased to 1% (w/v). Although the activities were measurable, the data obtained with these substrates are complicated by the relative insolubility of the anilides in water. Butyranilide appeared to be less soluble than acetanilide; this is probably responsible for the fact that butyranilide appeared to be less rapidly hydrolysed than acetanilide by some of the enzymes investigated.

Enzyme preparations. Aqueous homogenates of rat brain and liver were prepared with a glass Potter–Elvehjem homogenizer. As noted previously, the brain suspensions contain several esterases; all of the experiments described here are concerned only with the 'DINP-sensitive' esterase, which is responsible for about 70% of the total activity towards tributyrin and for all of the activity towards ethyl butyrate (Myers, 1956). The liver also contains several enzymes with the characteristics of ali-esterases (Falconer & Taylor, 1946; Fodor, 1946; Myers & Mendel, 1953; Myers, Kemp, Tol & de Jonge, unpublished results); the enzyme studied in the present investigation is responsible for more than 90% of the total activity towards tributyrin and for 99% of the total activity towards ethyl butyrate and phenyl butyrate (cf. Aldridge, 1954). This esterase would seem to be analogous to the ali-esterase from horse liver which was used by Schwert & Glaid (1952).

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The mycobacteria were supplied by Professor A. Charlotte Ruys, Laboratory of Hygiene, University of Amsterdam. The results obtained in growth experiments with *Mycobacterium phlei* and a similar strain of saprophytic mycobacteria designated as S3048 have been described previously (Ruys, 1954). Five other strains of saprophytic mycobacteria isolated from human-stomach washings were also selected for study; these strains have been designated as *Myco.* A, B, C, D and E in the present paper. (These strains are numbered as VII, XIV, IX, XII and XV respectively in the collection at the Laboratory of Hygiene.) The hitherto unpublished results obtained by Ruys in preliminary growth experiments with these strains are indicated in Table 1; it is hoped that more extensive results will be published elsewhere.

The ali-esterase content of the saprophytic mycobacteria seemed to be independent of the medium on which they were grown. Almost identical results were obtained with samples of the same strain grown in *bouillon*, the Dubos medium (cf. Dubos & Davis, 1946; Dubos & Middlebrook, 1947), the medium of Proskauer & Beck (1894; cf. Youmans & Karlson, 1947), or on the medium of Löwenstein (1930). The activities of the various strains towards tributyrin varied from 50 to 250 μ l. of CO₂/mg. dry wt. of bacteria/hr., but the values obtained were found to be characteristic of the strain in question rather than of the culture medium. The sensitivity of the ali-esterases to inhibition by E600 under the experimental conditions was also independent of the medium on which the bacteria had been grown.

The synthetic medium of Proskauer & Beck (1894) appeared to be the most convenient for culturing large amounts of mycobacteria; most of the experiments on the ali-esterases were carried out with mycobacteria which had been grown on this medium. The mycobacteria were harvested after they had been grown for 4 weeks at 37°; they were centrifuged down from the culture medium at 3000 rev./min. in a clinical centrifuge, washed with boiled water to remove the last traces of the culture medium together with any extracellular proteins, and resuspended in water with the aid of a Potter-Elvehjem homogenizer. The bacterial suspensions were stored at 0° until used. All experiments reported in this paper were carried out within

4 days after preparation of the bacterial suspension; however, the activity of the ali-esterases did not deteriorate appreciably within 2 weeks under these conditions.

RESULTS

The initial experiments on the inhibition of bacterial growth by organophosphorus compounds were carried out with *Mycobacterium tuberculosis* typus *humanus* H37Rv; the growth in the Dubos medium at 37° was delayed for several days in the presence of 10⁻⁵M E600 (Mendel *et al.* 1953; Ruys, 1954). DINP, the isopropyl analogue of E600, is considerably more active than E600 in preventing the growth of the tubercle bacilli (Ruys, unpublished results; see Table 1); this is presumably due to the greater stability of DINP in the Dubos medium. E600, in contrast to DINP, is slowly broken down by the A-esterase present in the human-serum albumin which is a component of the Dubos medium (Ruys, 1954; cf. Aldridge, 1953).

Mendel *et al.* (1953) showed that the ali-esterase of the tubercle bacilli is strongly inhibited by E600; however, accurate measurements of the esterase activity with the Warburg apparatus were not carried out owing to the high pathogenicity of these bacilli. Two strains of non-pathogenic mycobacteria, *Myco. phlei* and S3048, were therefore investigated by Ruys (1954; unpublished results). Although the ali-esterases of both of these strains were almost completely inhibited by 10⁻⁵M E600 within 30 min. at 37° in the Warburg apparatus, the rate of growth in the Dubos medium at 37° was not significantly affected by the same concentration of E600. It seemed possible that this finding might be explained by the rapid growth of these bacilli at 37°, since other data indicated that the concentration of E600 required to prevent the growth increases in

Table 1. *Effect of E600 and DINP on the growth of several strains of mycobacteria*

Mycobacteria were grown in the Dubos medium at 37° by the methods described by Mendel *et al.* (1953) and Ruys (1954). The relative rate of growth was estimated from the time taken to reach a visible turbidity due to the multiplication of the mycobacteria in the medium. This time does not provide an accurate criterion for comparing the rate of growth of different strains of mycobacteria, but does clearly demonstrate the effects of E600 and DINP when the rate of growth is compared with the control experiments which were carried out simultaneously with the same inoculum of a given strain of mycobacteria. The preliminary results given in this table are taken from unpublished studies of Ruys.

Strain of mycobacteria	Relative rate of growth (days to reach a visible turbidity in the Dubos medium)		
	Control	In the presence of 10 ⁻⁵ M E600	In the presence of 3 × 10 ⁻⁶ M DINP
<i>Myco. tuberculosis</i> H37Rv	3	16	(>85)
<i>Myco.</i> D	2½	(>40)	(>85)
<i>Myco.</i> E	3	(>85)	(>85)
<i>Myco.</i> C	4	(>50)	(>50)
<i>Myco. phlei</i>	1	1	—
<i>Myco.</i> A	3	4	3
<i>Myco.</i> B	2	3	2

proportion to the rate of growth (Mendel *et al.* 1953; Ruys, 1954). To test this possibility, five other strains of saprophytic mycobacteria were studied by Ruys (unpublished results). Her results showed that the growth of three of the five strains was strongly inhibited at 37° in the Dubos medium by 10^{-5} M E 600 or 3×10^{-6} M DINP; however, the growth of the other two strains was not affected by DINP and only slightly affected by E 600 (see Table 1). It seemed unlikely that these differences could be explained by the differences in the rate of growth. The ali-esterases of these saprophytic mycobacteria were therefore studied in detail to see if there was any correlation between the effect of the organophosphorus compounds on the growth and on the esterases of the mycobacteria.

Differentiation of the ali-esterases

In the course of the investigations on the inhibition of the esterases by various compounds, it became apparent that some of the mycobacteria must contain more than one ali-esterase with a measurable activity. The evidence for this conclusion in *Myco. phlei* and *Myco. B* is shown in Table 2; the results obtained with the strain designated as *Myco. E* are also included in this table for purposes of comparison.

With *Myco. E* the degree of inhibition of the ali-esterase activity increases progressively as the concentration of inhibitor is increased until the esterase activity appears to be completely inhibited (Table 2). These results fail to give any indication for the presence of more than one esterase in *Myco. E* which is capable of hydrolysing ethyl butyrate at a measurable rate. The same type of result was obtained with *Myco. C* and *Myco. D*.

With *Myco. phlei*, on the other hand, the inhibition of the esterase activity towards ethyl butyrate reaches a plateau when the concentration of DINP reaches 10^{-6} M; a further increase in the concentration up to 10^{-5} M DINP has little effect on the residual esterase activity (Table 2 and Fig. 1). The existence of this plateau value strongly suggests that *Myco. phlei* must contain at least two ali-esterases capable of hydrolysing ethyl butyrate, one of which is almost completely inhibited by 10^{-6} M DINP, whereas the other is not significantly inhibited before the concentration of DINP is increased above 10^{-5} M. A similar plateau value does not appear clearly when phenyl *N-n*-propylcarbamate is used as inhibitor, although the curve relating the esterase activity to the inhibitor concentration is somewhat flattened in the region of 10^{-6} M inhibitor (Fig. 1). However, a marked difference in the sensitivity to this carbamate does become evident when the DINP-resistant esterase activity is measured separately, in the presence of 10^{-5} M DINP to eliminate the activity of the DINP-

sensitive esterase. The DINP-resistant activity is almost completely inhibited by 10^{-6} M phenyl *N-n*-propylcarbamate, although the total activity in the absence of DINP is only slightly affected by the same concentration of this compound (Table 2 and Fig. 1). It is probable, therefore, that the DINP-resistant activity is due to an esterase which is relatively sensitive to inhibition by the carbamate derivative, whereas the DINP-sensitive activity is due to a second esterase which is less sensitive to the carbamate inhibitor. The DINP-sensitive enzyme, it might be noted, appears to be responsible for the major portion of the total esterase activity towards ethyl butyrate.

Myco. phlei also exhibits a low but measurable activity towards ethyl leucine and butyranilide (Table 3). The results obtained with these substrates confirm the above conclusions. Almost all of the activity of *Myco. phlei* towards ethylleucine is resistant to inhibition by 10^{-6} M phenyl *N-n*-propylcarbamate and sensitive to inhibition by 10^{-5} M DINP, whereas the activity towards butyranilide shows the reverse pattern, i.e. resistance to inhibition by 10^{-5} M DINP and sensitivity to 10^{-6} M carbamate (Table 3). The relationship between these results and those obtained with ethyl butyrate can be seen most clearly when the inhibition of the esterase activity is plotted against the inhibitor concentration, as in Fig. 1. The characteristics of the two esterases in *Myco. phlei* are readily apparent from this figure. Most of the activity towards ethylleucine is due to the DINP-sensitive esterase, whereas most of the activity towards butyranilide is due to the DINP-resistant esterase.

The results obtained with *Myco. B* can be analysed in the same way as described above for the esterases of *Myco. phlei*. The evidence again points to the presence of two esterases, but in this case the plateau value representing the borderline between the activities of the two esterases is observed with the inhibitor phenyl *N-n*-propylcarbamate and is not distinct with DINP (Table 2). When the activity towards ethyl butyrate is measured in the presence of 10^{-6} M phenyl *N-n*-propylcarbamate to eliminate the activity of the carbamate-sensitive esterase, it becomes apparent that the residual carbamate-resistant activity is relatively resistant to inhibition by DINP (Table 2). These results present a contrast to those obtained with *Myco. phlei*; the carbamate-resistant esterase activity of *Myco. phlei* is readily inhibited by DINP.

As with *Myco. phlei*, it is again found that the activities of *Myco. B* towards ethylleucine and butyranilide are, for the greater portion, due to separate enzymes. Almost all of the activity towards ethylleucine is resistant to inhibition by phenyl *N-n*-propylcarbamate in concentrations up to 10^{-4} M, whereas almost all of the activity towards

Table 2. *Effect of DINP and phenyl N-n-propylcarbamate on the hydrolysis of ethyl butyrate by mycobacteria*

Suspensions of mycobacteria were incubated for 30 min. at pH 7.4 and 37.5° with DINP, phenyl *N-n*-propylcarbamate, or both inhibitors simultaneously, in the concentrations given. Subsequently a small volume of substrate was added and the esterase activity measured manometrically.

Concn. of DINP (-log M)		Esterase activity towards ethyl butyrate (μ l. of CO ₂ /mg. dry wt./hr.)		
Concn. of -carbamate (-log M)		<i>Myco. phlei</i>	<i>Myco. B</i>	<i>Myco. E</i>
(No inhibitor)		120	75	115
8.0	—	104	75	105
7.0	—	60	73	56
6.0	—	24	59	11
5.0	—	22	28	1
4.0	—	11	9	0
3.0	—	2	2	0
—	9.0	—	74	104
—	8.0	118	69	58
—	7.0	111	45	12
—	6.0	98	19	3
—	5.0	72	15	1
—	4.0	22	14	0
—	3.0	4	7	0
5.0	—	22	—	—
5.0	8.0	20	—	—
5.0	7.0	13	—	—
5.0	6.0	1	—	—
5.0	5.0	0	—	—
—	6.0	98	19	—
8.0	6.0	83	19	—
7.0	6.0	38	19	—
6.0	6.0	2	18	—
5.0	6.0	1	16	—
4.0	6.0	—	8	—
3.0	6.0	—	2	—

Fig. 1. Effect of DINP and phenyl *N-n*-propylcarbamate on the ali-esterases of *Myco. phlei*. The values are calculated from the data given in Tables 2 and 3. Graph *A* demonstrates the effect of various concentrations of DINP on the total esterase activity towards butyranilide ($\times$), ethylleucine ($\blacktriangle$) and ethyl butyrate ($\circ$), and on the residual activity towards ethyl butyrate which remains in the presence of 10^{-6} M phenyl *N-n*-propylcarbamate ($\bullet$). Graph *B* shows the results obtained in parallel experiments with various concentrations of phenyl *N-n*-propylcarbamate; the same symbols are used but ($\bullet$) indicates the effect of the carbamate on the residual activity towards ethyl butyrate which remains in the presence of 10^{-6} M DINP (see Table 2).

Table 3. *Effect of DINP and phenyl N-n-propylcarbamate on the hydrolysis of ethylleucine and butyranilide by mycobacteria*

Suspensions of mycobacteria were incubated for 30 min. at pH 7.4 and 37.5° with DINP or phenyl *N-n*-propylcarbamate in the concentrations given. Subsequently a small volume of substrate was added and the esterase activity measured manometrically. The amount of mycobacteria used for each measurement was varied between 10 and 100 mg. dry weight depending on the magnitude of the esterase activity in question; the degree of inhibition produced did not appear to be markedly dependent on the amount of enzyme preparation employed.

Esterase activity (μ l. of CO₂/mg. dry wt./hr.)

Concn. of DINP (-log M)	Concn. of carbamate (-log M)	<i>Myco. phlei</i>		<i>Myco. B</i>		<i>Myco. E</i>	
		Ethyl- leucine	Butyr- anilide	Ethyl- leucine	Butyr- anilide	Ethyl- leucine	Butyr- anilide
(No inhibitor)		25.5	9.5	1.2	31.0	0.6	4.7
8.0	—	22.1	9.3	1.2	31.0	0.6	4.2
7.0	—	10.1	9.0	1.2	29.0	0.3	2.3
6.0	—	0.5	8.7	1.2	22.5	0.0	0.5
5.0	—	0.4	8.0	1.1	7.7	0.0	0.2
4.0	—	0.2	4.4	0.6	0.2	—	—
3.0	—	0.0	0.8	0.1	0.0	—	—
—	8.0	25.5	8.9	1.2	28.6	0.3	2.4
—	7.0	25.3	5.4	1.2	16.5	0.1	0.5
—	6.0	24.8	1.0	1.2	1.4	0.0	0.2
—	5.0	17.0	0.8	1.1	0.5	—	—
—	4.0	5.8	0.3	1.1	0.4	—	—
—	3.0	0.4	0.0	0.6	0.0	—	—

butyranilide is inhibited by a 100-fold smaller concentration of this compound (Table 3). The results obtained with *Myco. A* were qualitatively identical with those described above for *Myco. B*. With *Myco. A* and *Myco. B* as well as with *Myco. phlei*, therefore, a carbamate-sensitive enzyme is responsible for most of the activity towards butyranilide and a carbamate-resistant enzyme is responsible for most of the activity towards the ethyl ester of leucine. Although DINP rather than phenyl *N-n*-propylcarbamate was used to distinguish between the two esterases of *Myco. phlei* (Table 2), these enzymes will be referred to as the carbamate-sensitive and carbamate-resistant esterases to stress the analogy with the esterases of *Myco. A* and *Myco. B*.

It might be noted that the activity towards butyranilide is probably not due completely to the carbamate-sensitive esterase; conversely, the carbamate-sensitive esterase may also be able to hydrolyse the ethyl ester of leucine slowly. The results given for *Myco. phlei* in Table 3 and Fig. 1 indicate that the carbamate-resistant esterase is probably responsible for about 10% of the total activity towards butyranilide; the results obtained with ethylleucine are less decisive but possibly the carbamate-sensitive esterase may be responsible for about 2% of the total activity of *Myco. phlei* towards this ester. Similarly, the carbamate-resistant esterase of *Myco. B* is probably responsible for about 1.5% of the total activity towards butyranilide (Table 3).

The esterase present in *Myco. E* can also be

classified as a carbamate-sensitive enzyme (see Tables 2 and 3). This esterase appears to be responsible both for the very small activity of *Myco. E* towards ethylleucine and for the larger activity towards butyranilide (Table 3). The results obtained with *Myco. C* and *Myco. D* were again almost identical with those shown for *Myco. E* in Table 3.

The characteristics of the esterases described above are summarized in Table 4. This table shows the relative sensitivities of the esterases to inhibition by DINP and by phenyl *N-n*-propylcarbamate, together with the approximate pI_{50} values (negative logarithm of the molar concentration of inhibitor required to produce 50% inhibition of the esterase activity); the pI_{50} values were calculated from results similar to those given in Tables 2 and 3 by a graphical plot of the type shown in Fig. 1. The results obtained with E 600 in other experiments are also included in Table 4 for purposes of comparison.

The ali-esterases of the strains C, D and E exhibit similar characteristics with E 600 as well as with the other two inhibitors (Table 4). However, the remainder of the esterases described here fail to show any marked resemblances in their sensitivities to these inhibitors. For comparative purposes, we also studied the DINP-sensitive esterase of rat brain (cf. Myers, 1956) under the same experimental conditions, as well as the ali-esterase of rat liver which is responsible for the hydrolysis of ethyl and phenyl butyrates by this tissue (cf. Aldridge, 1954); the results shown in Table 4 do not provide any evidence of similarities between these enzymes.

Table 4. *Comparison of various ali-esterases in their sensitivity to three inhibitors*

Enzyme suspensions were incubated for 30 min. at pH 7.4 and 37.5° with the inhibitors before measuring the residual esterase activity; the type of result obtained is illustrated in Tables 2 and 3. The relative inhibition of the various esterases by a critical concentration of inhibitor is designated as complete or almost complete (+ +), partial (+), and slight or insignificant (0) inhibition. The pI_{50} values (-log. of the molar concentration of inhibitor required to produce 50% inhibition of the esterase activity) were estimated from the graphs shown in Fig. 1 and from similar plots of the results which were obtained with the other esterases indicated below. The values for the DINP-sensitive ali-esterase of rat brain were determined with 100 mg. fresh wt. of brain/5 ml. of reaction mixture, with tributyrin as substrate (cf. Myers, 1956). The pI_{50} values for the ali-esterase of rat liver are highly dependent on the concentration of enzyme; the values given below were determined with phenyl butyrate as substrate with 0.4 mg. fresh wt. of liver/5 ml. of reaction mixture.

Esterase		Relative inhibition by			pI_{50} values		
		10 ⁻⁶ M phenyl <i>N-n</i> -propyl- carbamate	10 ⁻⁶ M DINP	10 ⁻⁶ M E 600	Phenyl <i>N-n</i> -propyl- carbamate	DINP	E 600
Source	Classification						
<i>Myco. C</i>	Carbamate-sensitive	++	++	++	7.9	6.4	6.8
<i>Myco. D</i>	Carbamate-sensitive	++	++	++	8.1	6.1	7.0
<i>Myco. E</i>	Carbamate-sensitive	++	++	++	8.0	6.2	7.0
<i>Myco. phlei</i>	Carbamate-sensitive	++	0	+	6.8	4.0	5.1
<i>Myco. phlei</i>	Carbamate-resistant	0	++	++	4.5	7.2	7.8
<i>Myco. A</i>	Carbamate-sensitive	++	+	+	7.6	4.8	5.2
<i>Myco. A</i>	Carbamate-resistant	0	0	+	3.3	3.9	5.0
<i>Myco. B</i>	Carbamate-sensitive	++	+	++	7.0	5.5	6.8
<i>Myco. B</i>	Carbamate-resistant	0	0	++	3.1	4.0	6.1
Rat brain	DINP-sensitive	0	++	++	2.6	7.3	7.4
Rat liver	DINP-sensitive	+	++	++	5.7	8.9	9.0

Substrate specificity of the ali-esterases

On the basis of the differences between the esterases in their sensitivity to DINP and phenyl *N-n*-propylcarbamate (Table 4), it is possible to distinguish the activities of each of these esterases and consequently to study their substrate specificities. The results obtained in this way are shown in Table 5. To facilitate comparison of these results the esterases have been grouped according to their sensitivity to inhibition by phenyl *N-n*-propylcarbamate. The brain esterase could also be classified in the group of carbamate-resistant esterases (Table 4), whereas the ali-esterase of liver appears to occupy an intermediate position.

The results given in Table 5 show that the carbamate-sensitive esterases of *Myco. C*, *D* and *E* are not completely identical although they are very similar; on the basis of the results given in Tables 4 and 5, it does seem justified to consider these esterases as variations of one distinct enzyme. The carbamate-sensitive esterases of *Myco. phlei* and strains *A* and *B* also show some similarity to each other and to the esterases of strains *C*, *D* and *E* (Table 5), even though they differ considerably in their sensitivities to inhibition by E 600 and DINP (Table 4). The most striking differences in the relative substrate-specificity patterns are observed with the three phenyl-substituted esters; the rate of hydrolysis by the carbamate-sensitive esterases *Myco. phlei* and strains *A* and *B* decreases in the order ethyl β -phenylpropionate, ethyl phenylacetate, ethyl benzoate, whereas the rate of hydro-

lysis by the carbamate-sensitive esterases of strains *C*, *D* and *E* decreases in the reverse order, i.e. ethyl benzoate, ethyl phenylacetate, ethyl β -phenylpropionate.

The two carbamate-resistant esterases of *Myco. phlei* and *Myco. B* also show some resemblance to each other and to the ali-esterase of rat liver, although the liver esterase appears to hydrolyse all of the ethyl esters more efficiently than do the bacterial esterases in question (Table 5). In contrast to the carbamate-sensitive esterases, all of these enzymes exhibit an appreciable activity towards ethylleucine and little activity towards butyranilide. The DINP-sensitive esterase of rat brain failed to exhibit an appreciable activity towards either of these substrates (Table 5), and would therefore not seem to be related to any of the esterases found in the saprophytic mycobacteria with the possible exception of the carbamate-resistant esterase of *Myco. A*. This enzyme also exhibits an exceptional specificity pattern; it does not hydrolyse ethyl esters at an appreciable rate under the experimental conditions (Table 5.)

DISCUSSION

The present investigation was designed primarily to test the possible correlation between the effects of the organophosphorus compounds on the growth and on the ali-esterases of saprophytic mycobacteria. The classification of these ali-esterases was found to be more complicated than expected. However, the results show two general differences between the

ali-esterases of *Myco. phlei* and strains A and B, on the one hand, and the ali-esterases of strains C, D and E on the other hand.

(1) Two distinct esterases can be identified in the bacilli whose growth is relatively resistant to inhibition by E 600 and DINP, i.e. *Myco. phlei* and strains A and B. On the other hand, only one esterase could be identified in the bacilli whose

growth in the Dubos medium is readily inhibited by E 600 and DINP, i.e. strains C, D and E. The significance of this finding as an explanation for the effects of E 600 and DINP on the growth is open to some question. In the first place, it seems likely that the strains C, D and E might also contain other esterases but that the amounts would be too small to be distinguished with the substrates and

Table 5. Comparison of the relative substrate specificities of the various ali-esterases

Activity of the ali-esterases towards tributyrin is expressed as $\mu\text{l. of CO}_2/\text{mg. dry wt./hr.}$, and the relative activity towards the other substrates is calculated as a percentage of the activity towards tributyrin. The values in parentheses were too low to be determined accurately; omission of a value indicates that the activity was not measured. In one case with *Myco. A* and ethylleucine, it was not possible to obtain a reproducible value with different enzyme preparations. Most of the results on the relative substrate specificity of the DINP-sensitive, carbamate-resistant esterase of rat brain are taken from Myers (1956). The activities of each of these esterases were distinguished on the basis of their sensitivities to inhibition by DINP and phenyl *N*-propylcarbamate as indicated in Table 4.

Substrate	Activity of the carbamate-sensitive esterases					
	<i>Myco. phlei</i>	<i>Myco. A</i>	<i>Myco. B</i>	<i>Myco. C</i>	<i>Myco. D</i>	<i>Myco. E</i>
	($\mu\text{l. of CO}_2/\text{mg. dry wt./hr.}$)					
Tributyrin	20	35	120	95	48	48
	Relative activity (%)					
Tributyrin	100	100	100	100	100	100
Tripropionin	85	145	55	205	220	265
Triacetin	45	25	15	46	54	65
Phenyl butyrate	175	180	140	230	220	255
Phenyl propionate	150	225	95	310	310	390
Phenyl acetate	105	50	45	155	200	220
Ethyl butyrate	110	150	75	190	240	180
Ethyl β -phenylpropionate	45	60	10	35	58	35
Ethyl phenylacetate	20	40	8	51	65	50
Ethyl benzoate	(10)	20	(2)	62	75	60
Ethylleucine	(2)	(0)	(0)	1	1	1
Ethyl- β -phenylalanine	(0)	(0)	(0)	(0)	(0)	(0)
Butyranilide	40	10	26	9	12	10
Acetanilide	50	—	—	—	8	6

Substrate	Activity of the carbamate-resistant esterases			Activity of the DINP-sensitive esterases	
	<i>Myco. phlei</i>	<i>Myco. A</i>	<i>Myco. B</i>	Rat liver	Rat brain
	($\mu\text{l. of CO}_2/\text{mg. dry wt./hr.}$)				
Tributyrin	240	16	29	820	22
	Relative activity (%)				
Tributyrin	100	100	100	100	100
Tripropionin	90	90	105	120	165
Triacetin	35	65	30	20	23
Phenyl butyrate	205	240	400	340	225
Phenyl propionate	190	205	420	355	275
Phenyl acetate	60	160	125	140	195
Ethyl butyrate	40	(5)	50	330	10
Ethyl β -phenylpropionate	32	10	70	240	—
Ethyl phenylacetate	10	(0)	18	85	—
Ethyl benzoate	7	(0)	(1)	25	—
Ethylleucine	10	(?)	4	34	(0)
Ethyl- β -phenylalanine	1.5	(0)	3	13	(0)
Butyranilide	0.4	(0)	(1)	0.4	(>1)
Acetanilide	1	—	—	0.7	—

experimental methods used. Secondly, the carbamate resistant esterase of *Myco. phlei* is readily inhibited by E 600 and DINP; the carbamate-resistant esterase of *Myco. B* is also fairly sensitive to inhibition by E 600 (Table 4). This fact suggests that the presence of a carbamate-resistant esterase in these two strains of saprophytic mycobacteria could not explain the lack of effect of E 600 on their growth.

(2) The carbamate-sensitive esterases of *Myco. phlei* and the strains A and B act most efficiently on esters which have a larger acyl group than the esters which are hydrolysed most efficiently by the carbamate-sensitive esterases of the strains C, D and E. This difference is seen most clearly in the relative rates of hydrolysis of the three phenyl-substituted esters, as mentioned previously. It is possible, therefore, that the first group of esterases normally act on a substrate which has a larger acyl residue than the physiological substrate of the second group of esterases. The significance of this difference for the problem in question is uncertain; the remainder of the substrate-specificity pattern (Table 5) gives no indication that the general type of physiological substrate is essentially different.

The results of these experiments do not disprove the hypothesis that the effect of E 600 and DINP on the growth of the mycobacteria is related to the effect of these inhibitors on the ali-esterase activity (cf. Mendel *et al.* 1953). If we compare the esterase activities of the mycobacteria whose growth is resistant to E 600 and DINP with the esterase activities of the mycobacteria whose growth is readily prevented by these compounds under the conditions given in Table 1, we see that the general pattern of ali-esterase activity is the same in the members of each group, although differing from the general pattern of ali-esterase activity in the other group. This fact does suggest that the hypothesis may be correct.

It is not difficult to understand why the growth of the strains C, D and E could be prevented by the esterase inhibitors since the ali-esterases are readily inhibited by E 600 and DINP. Moreover, the esterases of these mycobacteria hydrolyse butyranilide and acetanilide fairly efficiently (Table 5); this finding suggests that these enzymes have a function in protein metabolism, i.e. in the hydrolysis of peptide or amide derivatives or both (cf. Michel *et al.* 1937). Under these circumstances it would not be surprising that these enzymes should prove to be essential for the normal growth of the mycobacteria.

However, a problem remains in connexion with the fact that E 600 and DINP have little effect on the growth of *Myco. phlei* and the strains A and B, even though certain of the ali-esterases concerned may be inhibited by these compounds. As indicated above, the presence of a second type of enzyme, the

carbamate-resistant esterases, in these strains of saprophytic mycobacteria does not seem to provide a sufficient explanation of this problem. In *Myco. B* both the carbamate-sensitive and the carbamate-resistant esterases are fairly sensitive to inhibition by E 600 (Table 4); both enzymes are almost completely inhibited by 10^{-5} M E 600 within 30 min. at 37.5°, although this concentration of inhibitor has little effect on the growth of *Myco. B* (Table 1). A similar situation is encountered with *Myco. phlei*. The carbamate-sensitive esterase is less readily inhibited by E 600 in this case; however, the carbamate-resistant esterase, which is responsible for the major portion of the activity towards tributyrin, ethyl butyrate and most of the other esters tested (Table 2 and 5), is highly sensitive to inhibition by E 600 and DINP (Table 4) and does not seem to be necessary for the growth of these bacilli (cf. Table 1). The results obtained with *Myco. A* are less significant since both esterases are moderately resistant to inhibition by E 600 and DINP (Table 4), and since the carbamate-resistant esterase exhibits a different substrate specificity (Table 5).

We might comment on the similarity in the substrate-specificity patterns of the ali-esterase of rat liver and the carbamate-resistant esterases found in *Myco. phlei* and *Myco. B*. The physiological significance of the liver esterase is also obscure; this enzyme does not seem to be of any immediate importance either for the oxidative metabolism of liver slices or for the regeneration of the liver after partial hepatectomy (cf. Myers & Mendel, 1953). Although we have little further information concerning the hydrolytic enzymes present in the mycobacteria, more extensive experiments have been carried out with liver preparations. Since the ali-esterase in liver seems to resemble the carbamate-resistant esterases in *Myco. phlei* and *Myco. B*, with regard both to their substrate specificities (Table 5) (cf. Cohen, Kushnick & Purdy, 1953) and their apparent dispensability for growth processes, it may be appropriate to discuss the enzymes in liver in more detail at this point.

Despite the fact that the ali-esterase of liver hydrolyses butyranilide fairly slowly (Table 5) and does not seem to exhibit any appreciable activity towards *N*-ethylbutyramide (Schwert & Glaid, 1952), we would suggest that this enzyme may have a function in protein metabolism. The results obtained in other experiments with selective esterase inhibitors indicate that the liver esterase does hydrolyse butyramide slowly (Myers, Kemp, Tol & de Jonge, unpublished results) and that it is probably identical with the liver amidase described by Bray, James, Thorpe & Wasdell (1950) and Bray, White & Wood (1955). In both cases, the enzyme activity shows an optimum between pH 8 and 9, and ex-

hibits the phenomenon of excess-substrate inhibition (Bamann, 1929, 1931; Murray & King, 1930; Bray *et al.* 1955). The activity of the amidase shows the following pattern, in decreasing order: caproamide, butyramide, acetamide, and γ -phenylbutyramide, β -phenylpropionamide, phenylacetamide, benzamide; this specificity pattern agrees well with the results reported above (Table 5), and by Sobotka & Glick (1934) for the esterase activity of liver. The amidase appears to be present in the same fraction of the liver as the enzyme which hydrolyses β -phenylalaninamide and leucinamide (Meister, Levintow, Greenfield & Abendschein, 1955). The similarity in the structure of these substrates suggests that the amidase activities towards β -phenylalaninamide and β -phenylpropionamide might well be due to the same enzyme; further experiments will be required to test the validity of this hypothesis.

The experimental results reported in the literature indicate that the liver contains at least two enzymes capable of hydrolysing leucinamide and β -phenylalaninamide. One of these enzymes, which is probably similar to the leucine aminopeptidase isolated from other tissues, is most active at a slightly acid pH, is strongly activated by manganese ions, does not hydrolyse amino acid esters and is not inhibited by organophosphorus compounds (cf. Smith, 1951*a, b*; Smith & Spackman, 1955). The other enzyme is active at more alkaline pH values and does not seem to require activating ions (cf. Errera & Greenstein, 1948; Meister *et al.* 1955). If, as we suggest, the latter enzyme is identical with the ali-esterase of liver, then it would be capable of hydrolysing esters as well as amide derivatives and would be sensitive to inhibition by organophosphorus compounds; similar properties have been demonstrated for other proteolytic enzymes such as trypsin and chymotrypsin (Neurath & Schwert, 1950).

Under these circumstances it is conceivable that complete inhibition of the liver ali-esterase by organophosphorus compounds might not have any obvious influence on the metabolism of this tissue, since the liver also contains another enzyme which is capable of hydrolysing the amino acid amides and which is not affected by the organophosphorus compounds. This seems at present to be the most logical explanation of the results mentioned above which we obtained previously in an investigation of the liver ali-esterase (Myers & Mendel, 1953). It is possible that an analogous situation might explain the results reported here for *Myco. phlei* and *Myco. B*.

The peptidases of mycobacteria have been studied by Roulet & Zeller (1945, 1948), but the connexion between the enzymes which they described and the ali-esterases described above is still a matter for speculation. If, as we suggest, the

carbamate-resistant and the carbamate-sensitive esterases of the mycobacteria may both be concerned with the hydrolysis of amides or peptides or both, a further study of the activity of these enzymes towards amino acid derivatives would be valuable in elucidating their physiological functions. It is to be expected that these investigations would shed further light on the reason for the differences observed in the effects of E600 and DINP on the growth of these mycobacteria.

SUMMARY

1. It had been observed that the growth of certain strains of saprophytic mycobacteria was prevented by organophosphorus compounds in low concentrations, whereas the growth of other strains of saprophytic mycobacteria was not affected under the same experimental conditions (Ruys, unpublished results). This observation provided an opportunity to investigate the suggested correlation between the effects of the organophosphorus compounds on the growth and on the esterase activities of the mycobacteria (Mendel *et al.* 1953).

2. Studies on the substrate specificities and sensitivities of these enzymes to inhibitors showed distinct differences between the ali-esterases of the two groups of mycobacteria. The significance of these differences is discussed.

3. The ali-esterases of the mycobacteria, like the ali-esterase of liver, are capable of hydrolysing a wide variety of esters, including amino acid esters as well as certain amides. It is suggested therefore that these enzymes might have a function in protein metabolism. On this basis it is possible to offer an explanation for the differences in the effects of the organophosphorus compounds on growth.

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REFERENCES

- Aldridge, W. N. (1953). *Biochem. J.* **53**, 117.
- Aldridge, W. N. (1954). *Biochem. J.* **57**, 692.
- Bamann, E. (1929). *Hoppe-Seyl. Z.* **183**, 149.
- Bamann, E. (1931). *Hoppe-Seyl. Z.* **194**, 1.
- Bray, H. G., James, S. P., Thorpe, W. V. & Wasdell, M. R. (1950). *Biochem. J.* **47**, 294.
- Bray, H. G., White, K. & Wood, P. B. (1955). *Biochem. J.* **59**, 167.
- Brooks, V. B., Ransmeier, R. E. & Gerard, R. W. (1949). *Amer. J. Physiol.* **157**, 299.
- Cohen, S., Kushnick, J. B. & Purdy, C. V. (1953). *J. Bact.* **66**, 266.
- Dubos, R. J. & Davis, B. D. (1946). *J. exp. Med.* **83**, 409.
- Dubos, R. J. & Middlebrook, G. (1947). *Amer. Rev. Tuberc.* **56**, 334.

- Errera, M. & Greenstein, J. P. (1948). *J. nat. Cancer Inst.* **3**, 71.
- Falconer, J. S. & Taylor, D. B. (1946). *Biochem. J.* **40**, 831.
- Fodor, P. (1946). *Nature, Lond.*, **158**, 375.
- Gerhardt, C. (1853). *Liebigs Ann.* **87**, 149.
- Kobezen, M. J., Metcalf, R. L. & Fuhuto, T. R. (1954). *J. agric. Food Chem.* **2**, 864.
- Löwenstein, E. (1930). *Dtsch. med. Wschr.* **56**, 1010.
- Meister, A., Levintow, L., Greenfield, R. E. & Abendschein, P. A. (1955). *J. biol. Chem.* **215**, 441.
- Mendel, B., Myers, D. K., Uyldert, I. E., Ruys, A. C. & de Bruyn, W. M. (1953). *Brit. J. Pharmacol.* **8**, 217.
- Michel, H. O., Bernheim, F. & Bernheim, M. L. C. (1937). *J. Pharmacol.* **61**, 321.
- Murray, D. R. P. & King, C. G. (1930). *Biochem. J.* **24**, 190.
- Myers, D. K. (1956). *Biochem. J.* **64**, 740.
- Myers, D. K. & Mendel, B. (1953). *Biochem. J.* **53**, 16.
- Myers, D. K., Schotte, A., Boer, H. & Borsje-Bakker, H. (1955). *Biochem. J.* **61**, 521.
- Neurath, H. & Schwert, G. W. (1950). *Chem. Rev.* **46**, 69.
- Proskauer, B. & Beck, M. (1894). *Z. Hyg. InfektKr.* **18**, 128.
- Roulet, F. & Zeller, E. A. (1945). *Experientia*, **1**, 122.
- Roulet, F. & Zeller, E. A. (1948). *Helv. chim. acta*, **31**, 1915.
- Ruys, A. C. (1954). *Leeuwenhoek ned. Tijdschr.* **20**, 145.
- Schroeter, G. (1909). *Ber. dtsch. chem. Ges.* **42**, 3356.
- Schwert, G. W. & Glaid, A. J. (1952). *J. biol. Chem.* **199**, 613.
- Smith, E. L. (1951a). *Advanc. Enzymol.* **12**, 191.
- Smith, E. L. (1951b). In *The Enzymes*, vol. 1, part 2, p. 793. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press.
- Smith, E. L. & Spackman, D. H. (1955). *J. biol. Chem.* **212**, 271.
- Sobotka, H. & Glick, D. (1934). *J. biol. Chem.* **105**, 199.
- Wislicenus, W. (1897). *Liebigs Ann.* **296**, 361.
- Youmans, G. P. & Karlson, A. G. (1947). *Amer. Rev. Tuberc.* **55**, 529.

Studies on Ali-esterases

6. SELECTIVE INHIBITORS OF THE ESTERASES OF BRAIN AND SAPROPHYTIC MYCOBACTERIA*

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Previous investigations have shown that the growth of *Mycobacterium tuberculosis* and certain strains of saprophytic mycobacteria can be prevented *in vitro* by certain organophosphorus compounds (Mendel, Myers, Uyldert, Ruys & de Bruyn, 1953; Ruys, 1954; Myers, Tol & de Jonge, 1957); it was suggested that the effect of these compounds on growth may be related to their ability to inhibit the ali-esterases of these mycobacteria. The organophosphorus compounds which were used in these experiments cannot be used for the same purpose *in vivo* since they are also potent inhibitors of true cholinesterase and are consequently highly toxic to animals. In an attempt to find more selective inhibitors of the ali-esterases, a series of compounds was synthesized and tested initially against the true cholinesterase and ali-esterase of rat brain. Some of the results obtained in these preliminary experiments have been reported previously (Myers, Mendel, Gersmann & Ketelaar, 1952; Mendel *et al.* 1953; Myers, 1953, 1954; Myers & Kemp, 1954).

In the present work a number of new inhibitory compounds were prepared and investigated as inhibitors of the ali-esterases of the saprophytic

mycobacteria and of the esterases of rat brain *in vitro*. The results obtained appear to offer valuable information concerning the factors which govern the selectivity of these inhibitors towards a given esterase.

EXPERIMENTAL

Determination of esterase activity. Esterase activity was measured by a manometric method in the Warburg apparatus at 37.5° and pH 7.4 in a medium containing 0.025 M-NaHCO₃ and saturated with CO₂ + N₂ (5:95, v/v). The reaction mixture was incubated for 30 min. at 37.5°, with or without added inhibitor, before adding a small volume of substrate to determine the residual esterase activity. The ali-esterase activities were measured with 0.2% (w/v) of tributyrin as substrate and the true cholinesterase activity of rat brain with 0.03 M acetyl-β-methylcholine. All values were corrected for the very slow spontaneous hydrolysis of the substrate in question under the same experimental conditions.

The enzyme preparations used were described in previous publications (Myers, 1956b; Myers *et al.* 1957); for comparative purposes, we have tested the true cholinesterase and ali-esterase of rat-brain homogenates, and the ali-esterases of five strains of saprophytic mycobacteria. Two of these strains, *Mycobacterium phlei* and *Mycob.* B, are not affected by the organophosphorus compounds in their growth at 37° in the medium of Dubos & Davis (1946), even though the ali-esterase activity is inhibited (Myers *et al.* 1957); the growth of the other three strains, which were

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