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The Hydrolysis of Esters of Phenol by Cholinesterases and Other Esterases

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In recent years it has been shown that cholinesterases of both types can catalyse the hydrolysis of many non-choline aliphatic esters (for literature see Whittaker, 1951). The primary object of the present investigation was to find out if cholinesterases can also hydrolyse non-choline esters possessing an aromatic nucleus.

A series of *p*-substituted phenyl acetates was selected for study. It was found that these esters are indeed hydrolysed rapidly by the purified cholinesterases of human erythrocytes and plasma and by cobra-venom cholinesterase. During the course of the work it became apparent, however, that, as with aliphatic esters, the hydrolysis of the aromatic esters by whole human erythrocytes and plasma could be accounted for only in part by the cholinesterase content of these preparations. Some additional studies were therefore made of the other esterases involved.

The hydrolysis of aromatic esters by blood and tissues has been the subject of several previous studies, but little attempt has been made to characterize the enzymes responsible for hydrolysing

them. The hydrolysis of short-chain aliphatic esters of *p*-nitrophenol (Huggins & Lapides, 1947; Huggins & Moulton, 1948) and of 2-naphthol (Nachlass & Seligman, 1949; Seligman, Nachlass & Mollomo, 1949) was attributed by these authors to aliphatic esterases (ali-esterases). Augustinsson (1948, 1950) studied the hydrolysis of acetylsalicylic acid, acetylsalicylcholine and salicylcholine by cholinesterase preparations derived from the blood, brain and tissues of various species, but no special precautions were taken to exclude other esterases. Zeller, Fleisher, McNaughton & Schweppe (1949) showed that preparations of snake venom and erythrocyte cholinesterase free from ali-esterase could hydrolyse phenyl acetate and *p*-nitrophenyl acetate, and that the hydrolysis was fully eserine-sensitive, the latter constituting good evidence that the cholinesterase was responsible. Diluted whole human plasma, however, split phenyl acetate very much faster than acetylcholine and the hydrolysis was but little depressed by eserine, a result consistent with that of Huggins & Lapides (1947) with *p*-nitrophenyl acetate. The hydrolysis of *m*-acetoxyphenyltrimethylammonium methosulphate by human serum and erythrocytes and rat brain was reported by Wescoe, Riker & Beach (1950). Again,

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no precautions were taken to exclude non-choline esterases.

Aldridge (1951*a, b*, 1953) has studied the hydrolysis of *p*-nitrophenyl acetate, propionate and butyrate by the sera and tissues of a wide range of species. In addition to any contribution made by cholinesterase, two other enzymes, named by him the A- and B-esterases, present in varying proportions in different sera, hydrolyse these substrates. The B-esterase, like the cholinesterases, is sensitive to toxic organophosphorus compounds; it hydrolyses butyrates faster than acetates. The A-esterase is not only insensitive to organophosphorus compounds; it has the remarkable property of hydrolysing at least one of them, diethyl *p*-nitrophenyl phosphate (E 600). The relation of these findings to our own work is discussed below.

MATERIALS AND METHODS

Substrates. Acetyl- β -methylcholine (MCh; β -acetoxypropyltrimethylammonium chloride) and acetylcholine (ACh) were obtained commercially; they were used either without further purification or after conversion to the perchlorates (Bell & Carr, 1947). Phenyl acetates were prepared from the corresponding phenol and acetic anhydride in the presence of aqueous NaOH (Chattaway, 1931). *p*-*tert*-Butylphenol was prepared as described by Liebmman (1881) and *p*-acetoxyphenyltrimethylammonium iodide (*p*-APTA) by warming *p*-dimethylaminophenyl acetate with methyl iodide.

Inhibitors. Eserine was obtained commercially; diisopropylphosphorofluoridate (DFP), $(\text{Pr}^{\text{iso}})_2\text{POF}$, was kindly provided by Dr D. R. Davies, Porton, and E 600 by Dr W. N. Aldridge, Carshalton. Commercial samples of these inhibitors were also used. The DFP was used without further purification; E 600 was freed from hydrolysis products and tetraethyl pyrophosphate by extracting an ethereal solution with Na_2CO_3 , drying and removal of ether *in vacuo*.

Human-erythrocyte aceto-cholinesterase*. Three preparations of differing degrees of purity were made from erythrocytes from time-expired or Kahn-positive human blood kindly supplied by the Oxford Blood Bank and the Blood Bank of the University of Cincinnati.

(A) **Crude preparation.** Laked cells were washed three times with 0.9% (w/v) NaCl and lysed in distilled water. This preparation contained, in addition to the aceto-cholinesterase, an aliphatic esterase (ali-esterase) and (as will be shown below) an aromatic esterase distinct from the other two esterases and capable of hydrolysing the phenyl acetates. Q_{MCh} (μl . acid liberated/mg. dry wt./hr. in presence of 30 mM-acetyl- β -methylcholine), 12.

(B) **Partially purified preparation.** Preparation A was brought to pH 5.9 and the precipitated stromata collected by centrifuging at 3000 g. They were washed with water and 0.1 M-phosphate buffer, pH 6.1, until the supernatant was almost colourless. After a final wash with water they were resuspended in water, adsorbed on to kieselguhr and eluted

with 0.2% (w/v) NaHCO_3 as previously described (Munter & Whittaker, 1950). Q_{MCh} , 380; purification, 32-fold; yield, 53%. This preparation hydrolysed tributyrin at less than 1% of the acetyl- β -methylcholine rate and was therefore free from ali-esterase by the criteria adopted in the previous specificity studies of Munter & Whittaker (1950).

(C) **Further-purified preparation.** Preparation B was further purified as follows. The cholinesterase was collected by centrifuging at 16000 g. for 30 min., dispersed in 0.2% (w/v) NaHCO_3 and incubated for 12 hr. at 38° with lysolecithin (King & Dolan, 1933). Inactive material was removed by centrifuging at 16000 g. for 30 min. The cholinesterase was recovered from the clear supernatant by precipitation at 50% saturation with $(\text{NH}_4)_2\text{SO}_4$, resuspended in water, dialysed and made 0.2% with NaHCO_3 . Q_{MCh} , 1060; purification, 83-fold, yield 18%.

Fig. 1. Hydrolysis of *p*-acetoxyphenyltrimethylammonium iodide by human-erythrocyte aceto-cholinesterase as a function of substrate concentration. Abscissa, negative logarithm of molar substrate concentration (*pS*); ordinate, activity as percentage of that at *pS*=0.

Cobra-venom aceto-cholinesterase. The cholinesterase of cobra venom has recently been shown to be a typical aceto-cholinesterase (Munter, 1951), and it is unaccompanied by ali-esterase. The same sample of venom was used as in the earlier experiments without purification.

Butyro-cholinesterase. Human plasma was used as the source of butyro-cholinesterase. Experiments were carried out with (a) whole plasma, (b) a partially purified preparation free from ali-esterase, obtained by following Strelitz's (1944) procedure to the end of stage 3, (c, d) Cohn plasma fractions IV-6 and IV-6.3, kindly supplied by Dr D. M. Surgenor, Harvard University. Dilution of the more active preparations was made in 0.2% (w/v) NaHCO_3 containing 1% (w/v) gum acacia.

Measurements of esterase activity. These were normally carried out at 37° and pH 7.4 by the Warburg manometric technique as modified by Ammon (1933) with the precautions and controls described in previous publications (Sturge & Whittaker, 1950; Munter, 1951). In some experiments the colorimetric method of Hestrin (1949) was used to follow the rate of disappearance of substrate. 100 μmoles of the phenyl esters or 30 μmoles of the choline

* For definition of 'aceto-cholinesterase' see Sturge & Whittaker (1950).

faster than acetyl- β -methylcholine. *p*-Methoxyphenyl acetate is the most rapidly hydrolysed; phenyl acetate follows, and *p*-iodophenyl and *p*-cresyl acetate are also split more rapidly than acetyl- β -methylcholine.

The aromatic esterase of erythrocytes. Some properties of the aromatic esterase were studied using unpurified laked erythrocytes (preparation A). The earlier results (Table 1) had indicated that this enzyme was relatively insensitive to DFP. This was confirmed in the experiments presented in Fig. 2. Increasing DFP concentrations caused a steep initial fall in the rate of phenyl acetate hydrolysis which was complete at $1\text{ }\mu\text{M}$, a concentration which, as shown by the MCh data inserted for comparison, completely inhibits cholinesterase activity. Thereafter, little further fall in the rate of phenyl acetate hydrolysis occurred even when the DFP concentration was increased 100-fold. This residual DFP-

Fig. 2. Effect of eserine (continuous lines) and DFP (broken lines) on hydrolysis of phenyl acetate ($\circ$), acetyl- β -methylcholine (+) and tributyrin (Δ) by laked human erythrocytes. Ordinates, activity expressed as a percentage of uninhibited activity; abscissa, negative logarithm of molar inhibitor concentration (pI).

insensitive activity must be attributed to a DFP-insensitive aromatic esterase. The fact that tributyrin hydrolysis was almost completely inhibited by $1\text{ }\mu\text{M}$ -DFP again shows that the aromatic esterase is distinct from the aliphatic esterase hydrolysing tributyrin.

In similar experiments with eserine, the cholinesterase is completely inhibited at $1\text{ }\mu\text{M}$ -inhibitor concentration while the phenyl acetate hydrolysis is only partially inhibited at many times this concentration. It should be noted, however, that the aliphatic esterase is also unaffected by this inhibitor, and that the portion of the phenyl acetate hydrolysis insensitive to $1\text{ }\mu\text{M}$ -eserine is greater than the portion insensitive to $1\text{ }\mu\text{M}$ -DFP. This suggests that a small portion of the phenyl acetate hydrolysis is due to the aliphatic esterase. Taking the activity

abolished by $1\text{ }\mu\text{M}$ -eserine as that due to the cholinesterase and the activity abolished by $1\text{ }\mu\text{M}$ -DFP as that due to cholinesterase plus ali-esterase, the contribution made by the three esterases to the hydrolysis of each of the three substrates may be calculated. The results are presented in the form of block diagrams in Fig. 3. The portion of the total phenyl acetate hydrolysis attributed to cholinesterase in this figure, when expressed as a percentage of the acetyl- β -methylcholine hydrolysis, gives a value identical with that obtained with purified cholinesterase (Table 2).

Fig. 3. Contribution made by cholinesterase (ChE), aromatic esterase (AromE) and aliphatic esterase (AliE) of laked human erythrocytes to hydrolysis of phenyl acetate, acetyl- β -methylcholine and tributyrin. The percentage contribution made by each enzyme to the total hydrolysis of each substrate is indicated within the histograms.

It is interesting to note that about 16% of the total phenyl acetate hydrolysis is attributed to the aliphatic esterase; since the total rate of hydrolysis of phenyl acetate is so much higher than that of tributyrin, phenyl acetate is actually split nearly three times as fast as tributyrin by this esterase. By contrast, the results with tributyrin show that this substrate is attacked at less than 1% of the rate of phenyl acetate by the aromatic esterase. *iso*Amyl acetate and *n*-hexyl acetate are also split by it at a negligible rate (Table 5).

The specificity of the aliphatic and aromatic esterases towards other phenyl acetates was next studied; the results are presented in Table 4, and were obtained as follows. The aromatic esterase figures were obtained as before by measuring the

rate of hydrolysis of the esters by laked erythrocytes in the presence of $1\ \mu\text{M}$ -DFP. The figures for the aliphatic esterase were obtained by subtracting from the DFP-sensitive portion of the total hydrolysis the contribution made by the cholinesterase. This was calculated from the specificity results obtained with preparation *C* (Table 2) and the known cholinesterase activity of the preparation. The results for tributyrin are inserted for comparison.

Table 4. *Specificity of the aromatic and aliphatic esterases of human erythrocytes*

Ester $p\text{-X}\cdot\text{C}_6\text{H}_4\text{OAc}$	Rate of hydrolysis of ester (as % of phenyl acetate hydrolysis) by	
	Aromatic esterase	Aliphatic esterase
X = Cl	149	7
Br	134	2
I	175	18
Me	90	4
MeO	111	5
tert.-Bu	32	7
Me_3N^+	130	0
Tributyrin	<1	35

Table 5. *Hydrolysis of aliphatic and aromatic esters by human erythrocyte-cholinesterase preparations*

Substrate	Rate (% MCh)		
	Laked erythrocytes (A)	Partially purified preparation (B)	Further- purified preparation (C)
Tributyrin	22	<1	<1
<i>n</i> -Hexyl acetate	—	22	21
<i>iso</i> Amyl acetate	—	—	70
Phenyl acetate	372	300	192
<i>p</i> -Cresyl acetate	270	253	157

Although some error may attach to results obtained in this indirect way, they serve to show that the aromatic esterase is able to split *p*-substituted phenyl acetates readily, often at a rate surpassing that of phenyl acetate itself. By contrast, *p*-substitution appears to exert an unfavourable effect on hydrolysis by the aliphatic esterase, though the rates of hydrolysis of some of the aromatic esters are by no means negligible when compared with that of tributyrin.

A comparison of the relative rates of hydrolysis of tributyrin and phenyl acetate by the three erythrocyte preparations reveals, in the light of Fig. 3, that the preparation of *B* from *A* and of *C* from *B* correspond to the stepwise removal of the aliphatic and aromatic esterases, respectively. It will be seen in Table 5 that removal of ali-esterase activity from *A* resulted in a fall in the phenyl acetate hydrolysis from 372 to 300 % and in the *p*-cresyl

acetate hydrolysis from 270 to 253 %. A preparation containing all of the cholinesterase of *A* and 85–86 % of the aromatic esterase would have just this activity.

The presence of a second carboxylic esterase in an erythrocyte cholinesterase preparation, judged enzymically homogeneous by previous criteria, raises the question as to whether the earlier specificity results of Adams & Whittaker (1948), Adams (1949) and Mounter & Whittaker (1950) can be considered reliable. The relative rates of hydrolysis of *iso*amyl acetate and *n*-hexyl acetate by the aromatic esterase-free preparation *C* was therefore determined; the results (Table 5) were identical with those obtained earlier.

The equivocal nature of the results given by the competition experiments should be noted. As seen in Table 3, the rate of hydrolysis of a mixture of phenyl acetate and acetyl- β -methylcholine by unpurified laked erythrocyte lies between the rates of hydrolysis of phenyl acetate and acetyl- β -methylcholine separately. This type of result in a competition experiment has often been taken as evidence that both substrates are competing for the same enzyme (cf. Augustinsson, 1950); it clearly does not exclude the possibility that one or both of the pair of competing substrates are also being split by enzymes which do not attack the other substrate. Quantitatively, the results of Table 3 reflect the stepwise elimination of the two non-choline esterases from the laked erythrocytes, but qualitatively, the same type of result was obtained with all three preparations.

Cobra venom

The relevant results presented in Table 2 show no evidence for the presence of an eserine-insensitive aromatic esterase in the crude venom. The absence of ali-esterase has already been noted (Mounter, 1951). All phenyl esters tested were hydrolysed at an appreciable rate and the hydrolysis was inhibited 97–100 % by $10\ \mu\text{M}$ -eserine, a concentration which inhibits the cholinesterase by the same amount (Mounter, 1951). The rates, relative to acetyl- β -methylcholine, were 28–77 % lower than those obtained with the erythrocyte cholinesterase and there were two differences in the order in which the substrates were hydrolysed. The lower rate of hydrolysis may be related to the sharper aliphatic specificity already noted for this enzyme (Mounter, 1951).

Plasma

Whole human plasma hydrolysed phenyl acetates at a high rate relative to acetylcholine but this hydrolysis was inhibited only a few per cent (Table 2; Fig. 4) by $1\ \mu\text{M}$ -DFP and $10\ \mu\text{M}$ -eserine (cf. Huggins & Lapides' (1947) results with *p*-nitrophenyl esters).

Table 6. *Relative rates of hydrolysis of p-substituted phenyl acetates by human-plasma butyryl-cholinesterase preparations of increasing purity*

Preparation	Q_{ACh}	Rate of hydrolysis (% ACh) of		
		Phenyl acetate	tert.-Butylphenyl acetate	p-APTA
(b) Oxford preparation	150	91	15	95
(c) Harvard plasma fraction IV-6	975	92	12.5	—
(d) Harvard plasma fraction IV-6-3	140 000	90	10.5	89*

* Perchlorate.

Partially purified butyryl-cholinesterase (preparation b) also hydrolysed these esters (Table 2) but at much lower rates relative to acetylcholine and the hydrolysis was now fully sensitive to $10 \mu\text{M}$ - eserine. Two other human-plasma butyryl-cholinesterase preparations (c and d) of high purity hydrolysed the esters at essentially the same relative rates (Table 6) as the less pure preparation (b). It is thus clear that a typical butyryl-cholinesterase can hydrolyse these aromatic esters though it accounts for only a few per cent of the hydrolysis of the esters by whole plasma. If the results of Table 2, col. 2, are expressed relative to the maximum rate of hydrolysis of acetylcholine by erythrocytes ($ACh/MCh=3$) the relative rates of hydrolysis of the aromatic esters by the two cholinesterases are found to be of the same order of magnitude (16–91%).

The aromatic esterase of plasma. It has been known for some time that human plasma contains a carboxylic esterase (or esterases) in addition to the butyryl-cholinesterase. Adams & Whittaker (1949a) reported the presence of an esterase insensitive to $0.1 \mu\text{M}$ -DFP which accounted for about 10% of the tributyrin hydrolysis and 20% of the triacetin hydrolysis of their plasmata. Myers & Mendel (1949) showed that human plasma contains an eserine-insensitive esterase which hydrolyses ethyl chloroacetate. The non-choline esterase has however been regarded as a minor constituent of human plasma in contrast to horse plasma which has a high 'ali-esterase' activity (for literature see Sturge & Whittaker, 1950). The high rate of hydrolysis of certain aromatic esters, unaccounted for by the cholinesterase content of the plasma, suggests that a second esterase distinct from the cholinesterase may, after all, be a major constituent of human plasma. It also raises the question as to the relation of the various non-choline esterase activities of plasma to the esterases of erythrocytes and to each other.

In an attempt to answer some of these points, we have studied the effect of DFP and eserine on the hydrolysis of phenyl acetate in the same way as with laked erythrocytes. As will be seen in Fig. 4, phenyl acetate hydrolysis is depressed to a small and approximately equal extent by concentrations of DFP and eserine which are fully inhibitory to

acetylcholine hydrolysis. The DFP- and eserine-sensitive portions of the hydrolysis are in good agreement with the value calculated from the cholinesterase content of the plasma and the phenyl acetate/acetylcholine activity ratio of purified plasma cholinesterase. The esterase mainly responsible for the hydrolysis of phenyl acetate in whole plasma is thus a DFP- and eserine-insensitive enzyme similar to the aromatic esterase of erythrocytes. Tributyrin hydrolysis, by contrast, was fully accounted for by the cholinesterase content of

Fig. 4. Effect of eserine (continuous lines) and DFP (broken lines) on hydrolysis of phenyl acetate ($\circ$), acetylcholine ($\times$) and tributyrin (Δ) by human plasma. Units as in Fig. 2.

the plasma: its inhibition closely paralleled that of acetylcholine, being negligible at an inhibitor concentration of $1 \mu\text{M}$ or more, and the tributyrin/acetylcholine activity ratio (46%) was equal to that of purified butyryl-cholinesterase (Adams & Whittaker, 1949b). This shows that the aromatic esterase of plasma, like that of erythrocytes, has little or no capacity to hydrolyse tributyrin, and that the eserine-insensitive DFP-sensitive aliphatic esterase of erythrocytes was not present. It is not inconceivable, however, that the aromatic esterase has some action on aliphatic esters, so that the DFP-insensitive tributyrin and triacetin hydrolysis noted by Adams & Whittaker (1949a) may have been due to an unusually high aromatic esterase activity in their plasmata.

*Relation of the esterases of human blood
to the A- and B-esterases*

The work so far described has revealed the existence in human blood of two esterases, in addition to the cholinesterases, which participate in the hydrolysis of aromatic esters. One, present in both cells and plasma, is uninhibited by high concentrations of DFP and has little or no action on the typical aliphatic ester, tributyrin. The other, present only in erythrocytes, is sensitive to DFP and

Fig. 5. Effect of E 600 (continuous lines) and DFP (broken lines) on hydrolysis of phenyl acetate by human erythrocytes and plasma. Units as in Fig. 2.

hydrolyses tributyrin faster than most aromatic esters tested. Both esterases are relatively insensitive to eserine. Our aromatic esterase somewhat resembles the A-esterase recently described by Aldridge (1951*a, b*, 1953) both in hydrolysing aromatic esters and in being insensitive to DFP; the aliphatic esterase may be related to the B-esterase which is sensitive to toxic organo-phosphorus compounds. Little is known of the ability of the A and B enzymes to hydrolyse aliphatic substrates, but like our esterases they are eserine-insensitive. A further point of significance is that the A enzyme alone occurs in human plasma (Aldridge, 1953).

In order to explore these resemblances further, three salient properties of the A- and B-esterases were selected for comparison; their differential sensitivity to E 600, their differing acyl group optima and the pH of optimum activity.

E 600 sensitivity. Fig. 5 shows that the effect of E 600 on the hydrolysis of phenyl acetate by erythrocytes and plasma closely parallels that of DFP. The hydrolysis consists of E 600-sensitive and E 600-insensitive portions approximately equal in magnitude to the DFP-sensitive and -insensitive activities. It follows that the esterases present in these preparations are similarly affected by DFP and E 600, i.e. the aromatic esterase, like the A-esterase, is insensitive to E 600 while the aliphatic esterase, like the B-esterase, is E 600-sensitive.

Acetate/butyrate activity ratios. Another distinguishing feature of the A- and B-esterases is the different relative rates at which they split *p*-nitrophenyl acetate and butyrate. The rate of hydrolysis of phenyl butyrate by the three esterases was measured as before using $1\mu\text{M}$ -DFP to inhibit aliesterase and cholinesterase and $1\mu\text{M}$ -eserine to inhibit cholinesterase. With the erythrocyte preparation, triacetin was also used as a substrate. The acetate/butyrate ratios were then evaluated and are presented in Table 7. It will be seen that the aromatic esterases of erythrocytes and plasma hydrolysed phenyl acetate much faster than phenyl butyrate; the acetate/butyrate ratio was in fact higher than that found by Aldridge (1951*b*) for the A-esterase of human plasma and esters of *p*-nitrophenol.

Triacetin was hydrolysed at about 15% of the phenyl acetate rate by erythrocyte aromatic esterase. Since tributyrin is hydrolysed at less than 1% of the phenyl acetate rate, the acetate/butyrate ratio for triglycerides must be greater than 15. This suggests that the aromatic esterase may after all have appreciable activity on some aliphatic esters. There is evidence that the A-esterase can slowly hydrolyse ethyl esters (Aldridge, 1951*b*).

The acetate/butyrate activity ratio for the aliphatic esterase of erythrocytes varied, being above unity for the phenyl esters and just less than unity for glyceryl esters. It was, however, much lower than the ratios found with the aromatic esterase. The B-esterases invariably had acetate/butyrate ratios well below unity for *p*-nitrophenyl esters (Aldridge, 1951*b*).

Optimum pH. The optimum pH of the A-esterase is 7.5, with E 600 as substrate (Aldridge, 1951*b*).

Table 7. *Acetate/butyrate activity ratios for non-choline esterases of human blood*

Esters	Acetate/butyrate activity ratio			
	Erythrocyte		Plasma	
	Aliphatic esterase	Aromatic esterase	Aromatic esterase	A-esterase*
Phenyl	4.4	22	30	—
Glyceryl	0.98	> 15	—	—
<i>p</i> -Nitrophenyl	—	—	—	8

* Aldridge (1951*b*).

The optimum pH of the aromatic esterase was determined using plasma incubated with $1\mu\text{M}$ - eserine as the source of the enzyme and phenyl acetate as substrate. The reaction was carried out at different pH values in the presence of 0.05M -phosphate, and was followed by Hestrin's (1949) colorimetric method for carboxylic esters. As seen in Fig. 6, the optimum pH lies close to 7.5.

Fig. 6. Effect of pH on hydrolysis rate of phenyl acetate by the aromatic esterase of human plasma.

Three lines of evidence, insensitivity to E 600 and DFP, the acetate/butyrate ratio and the optimum pH thus suggest that our aromatic esterase and Aldridge's A-esterase are identical.

DISCUSSION

The ability of two aceto-cholinesterases and one butyro-cholinesterase to hydrolyse aromatic esters, as exemplified by a series of *p*-substituted phenyl acetates, has been demonstrated. The high rate of hydrolysis of these esters appears to be an exception to the general rule that the rate of hydrolysis of non-choline esters is determined by their degree of structural resemblance to the optimum choline ester. Exceptions to this rule have already been noted and discussed (Mounter & Whittaker, 1950; Whittaker, 1951). The benzene ring is a highly polarizable structure and may be expected to confer an affinity upon these esters greater than that of structural analogues in the aliphatic series. Further, the non-enzymic rate of hydrolysis of all these compounds is higher than for simple aliphatic esters and this may be related to a greater instability of the enzyme-substrate complex.

Two other esterases have been found in human

blood, but not in cobra venom, which also hydrolyse phenyl acetate. Both of these are insensitive to eserine but differ in their sensitivity to toxic organophosphorus compounds. One of them appears to be identical with the well known ali-esterase of human erythrocytes and hydrolyses all the esters except phenyl acetate less rapidly than tributyrin. This enzyme is absent from the plasma. The other esterase, here referred to as the aromatic esterase, is present in both erythrocytes and plasma and is characterized both by the low rate at which it attacks glyceryl esters and its high resistance to DFP and E 600. These properties, and the fact that it remained in an erythrocyte cholinesterase preparation which had been freed from ali-esterase, clearly distinguished it from the later enzyme. Proof of the identity of the plasma and erythrocyte aromatic esterases must await their isolation, but if not identical they are at any rate closely similar. Several lines of evidence suggest that the aromatic esterase is probably identical with the E 600-insensitive esterase recently described by Aldridge and named by him the A-esterase. In spite of its low rate of hydrolysis of aliphatic esters, its presence could probably account for all of the so-called aliphatic esterase activity of plasma. The ali-esterase is less certainly but quite possibly to be identified with Aldridge's B-esterase. If this is true our concept of ali-esterase activity will have to be revised. The so-called ali-esterases may emerge as a group of relatively non-specific enzymes which are not limited to attacking aliphatic esters, or even, in the case of at least one member of the group, to carboxylic esters.

The physiological role of these esterases remains obscure. It is possible, however, that they play a part in destroying hormones or drugs whose activity depends on an ester linkage and this possibility may prove relevant to our understanding of the mode of action of such substances.

SUMMARY

1. The ability of two aceto-cholinesterases and one butyro-cholinesterase to catalyse the hydrolysis of a series of *p*-substituted phenyl acetates has been demonstrated.

2. These esters are also attacked by an aromatic esterase present in human erythrocytes and plasma but not in cobra venom.

3. This enzyme attacks aliphatic esters slowly and is not affected by relatively high concentrations of diisopropyl phosphorofluoridate or diethyl *p*-nitrophenyl phosphate. It is probably identical with the recently described A-esterase.

4. The well-known ali-esterase of human erythrocytes also appears to hydrolyse the aromatic esters, but in general less rapidly than tributyrin.

It can be separated from the aromatic esterase and is further distinguished from it by its sensitivity to toxic organophosphorus compounds.

5. Phenyl esters are thus hydrolysed by at least five esterases in human blood: erythrocyte aceto-cholinesterase, plasma butyryl-cholinesterase, erythrocyte ali-esterase, erythrocyte aromatic esterase

and plasma aromatic esterase. The last two enzymes are either identical or closely similar.

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The Lipids of Fish

3. THE ACETONE-INSOLUBLE FRACTION OF AN ACETONE EXTRACT OF THE FLESH OF THE HADDOCK

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The first paper in this series (Lovern, 1953) described the preparation of successive lipid extracts from the flesh of the haddock, using solvents of increasing effectiveness, and the second paper (Lovern & Olley, 1953) dealt with the acetone-soluble lipids present in the total material extracted by acetone. It was shown that this crude extract also contained much lipid which, after partial purification by reprecipitation procedures, was practically insoluble in acetone at 0°. The present paper describes the examination of this subfraction of the total acetone extract, designated fraction C in the previous paper.

ANALYTICAL METHODS

In general the analytical methods used were those described in the previous paper (Lovern & Olley, 1953), but some discussion seems desirable here of N, P and glycerophosphate

determination. Mention should also be made of the difficulty of accurate colorimetric determination of low concentrations of plasmalogens (Klenk & Friedrichs, 1952). We have found it preferable, in suitable cases, to determine plasmalogens by direct isolation and weighing of the plasmals, by the procedure of Feulgen, Imhäuser & Behrens (1929), having first removed other unsaponifiable matter by ether extraction of an alkaline hydrolysate of the lipid fraction. We have confirmed that such plasmal extracts give the expected intense aldehyde reaction with the fuchsin reagent, and agreement between the direct colorimetric estimation and the gravimetric estimation is satisfactory.

Nitrogen

Reference was made in the previous paper to the apparent loss of both N and P during hydrolysis of phospholipins. This phenomenon was again encountered with all the fractions described in the present paper. So far as N is concerned, the Kjeldahl digestion mixture (Olley, 1953) was selected because it was found to give quantitative ammonia