losses were negligible in extracts prepared with the 16-carbon compounds (with the exception of hexadecylpyridinium derivative). Generally, the keeping properties of extracts improved as the length of the alkyl chain was increased.

3. When a batch of retinae was repeatedly extracted with small volumes of a given concentration of an extracting-agent solution, the first extract usually contained much less rhodopsin than its successors. These extracts all had the same rhodopsin content (provided the retinae did not become exhausted). This suggests that initially some of the extracting agent is 'inactivated' owing to its combination with non-photosensitive retinal proteins. The calculated amount of extracting agent so inactivated was found to be constant for a given extracting agent. As soon as this combination has been completed, rhodopsin is extracted in quantities proportional to the extracting-agent concentration.

4. The slope of the straight line obtained by plotting extracting agent concentration against rhodopsin concentration varies for different extracting agents. The molar ratio of extracting agent:rhodopsin increases from 330 for the 16-carbon hexadecylammonium chloride to 48358 for the 5-carbon amylammonium chloride. The extraction curves for the 16-carbon quaternary compounds are identical with that of hexadecylammonium chloride. The slope of the dodecamethylene-1:12-diammonium dichloride extraction curve is nearly half that of the corresponding monoammonium compound, dodecylammonium chloride.

5. It is suggested that the cationic extracting agent forms a water-soluble complex with rhodopsin. The reactions involved are as follows: (a) The extracting-agent cations form a single layer over the rhodopsin peptide chain. This is due to the electrostatic attraction between the polar head group of the cation, and oppositely charged groups on the peptide chain. The alkyl chains of this layer point towards the aqueous phase. (b) A second layer of cations

amphipathically adsorbed on to the first produces a water-soluble complex. The addition of an extra terminal NH_3 to dodecylammonium chloride (thus forming dodecamethylene-1:12-diammonium chloride) reduces the molar ratio of extracting agent:rhodopsin by nearly half. This is attributed to the fact that the ion has become hydrophilic at both ends of the alkyl chain, and hence double-layer formation is not necessary for the formation of a water-soluble complex.

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The Hydrolysis of Indoxyl Esters by Esterases of Human Blood

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A new method for the localization of esterases in animal tissues by a histochemical staining process, using indoxyl esters as substrates, has recently been described by Barnett & Seligman (1951) and independently by Holt (1952). The indoxyl esters are hydrolysed by tissue esterases to indoxyl, which is subsequently oxidized to indigo, forming an in-

tensely coloured visual indicator of the sites of enzymic activity.

Detailed and characteristic histological pictures are obtained by a modification of this procedure, developed in these laboratories, which employs certain ring-substituted indoxyl esters as substrates (Holt & Withers, 1952; Holt, 1954). Recently, the

results of a systematic study of some fundamental aspects of histochemical staining procedures have been reviewed and a description is given of the development of a much improved indoxyl method for esterases which appears to have a precision of about 0.5–1.0 μ (Holt, 1956). To interpret fully the results of such histochemical procedures, it is essential to know which esterases are capable of hydrolysing the substrates used.

The indoxyl esters show structural analogies to acetylcholine (Holt, 1952) as well as to various aromatic esters such as phenyl and naphthyl esters. Mounter & Whittaker (1953) found that phenyl acetate is rapidly hydrolysed by aliphatic and aromatic esterases as well as by cholinesterases, whereas the naphthyl esters are generally regarded as being substrates for esterases rather than cholinesterases (Nachlas & Seligman, 1949; Gomori, 1953). It is therefore very likely that histochemical procedures which use indoxyl esters as substrate demonstrate the combined activity of several esterases at any given site.

Since pure enzyme preparations are not available the method of differential inhibition is generally used to study the contribution made by the various esterases of a crude enzyme preparation towards the hydrolysis of a given substrate (Aldridge, 1953*a*; Mounter & Whittaker, 1953). This technique is based on the finding that cholinesterases are inhibited by low concentrations of both eserine and organophosphates; a second type of esterase, aliphatic esterase, is inhibited only by organophosphates, and a third type of esterase, aromatic esterase, is not inhibited by either eserine or organophosphates.

The method of differential inhibition has now been used to study the enzymic hydrolysis of some unsubstituted indoxyl esters and also of α -naphthyl acetate, another currently used histochemical substrate for esterases.

Unsubstituted indoxyl esters have been chosen as model substrates in this initial study because of their greater solubility compared with that of the substituted derivatives at present used in these laboratories in histochemical staining procedures. This greater solubility allows the use of a convenient manometric procedure for the study of esterase activity. Human red cells and plasma were chosen as the sources of esterases, and the results presented below show that the indoxyl esters and α -naphthyl acetate, like phenyl esters, are hydrolysed by the two cholinesterases, and by the aliphatic and aromatic esterases from these sources.

MATERIALS AND METHODS

Substrates. The substrates used were indoxyl and phenyl acetates, propionates and butyrates, acetylcholine chloride, propionyl and butyrylcholine iodides and α -naphthyl

acetate. The indoxyl esters, propionyl and butyrylcholine iodides and α -naphthyl acetate were provided by Dr S. J. Holt and were of analytical purity. The phenyl esters were obtained commercially (B.D.H. Ltd.) and purified by the method used by Aldridge (1954). Acetylcholine chloride was obtained from Roche Products Ltd.

Inhibitors. Inhibitors used were eserine sulphate (Burroughs Wellcome and Co.); di-isopropyl phosphorofluoridate (DFP) and *p*-chloromercuribenzoic acid.

Enzyme preparations. The sources of mixed esterases were heparinized human plasma and human red cells which had been twice washed with 0.9% NaCl. Highly purified enzymes were bovine red-cell cholinesterase (Winthrop Stearns Inc.) and human plasma fraction IV-6.3 which had been prepared in the late Dr Cohn's laboratory.

Measurement of esterase activity. Enzymic activity was determined manometrically in the Warburg apparatus at 37° in a medium of 0.025 M-NaHCO₃, containing 0.1% of crystallized bovine-plasma albumin (Armour Laboratories) and saturated with N₂ + CO₂ (95:5); pH 7.5.

As the indoxyl esters have a low solubility in aqueous media it is not possible to obtain sufficiently concentrated stock solutions in 0.025 M-NaHCO₃. The esters are, however, far more soluble in organic solvents and of those investigated ethanol proved to be the most satisfactory. All the substrates except the choline esters were therefore dissolved in absolute ethanol and 0.15 ml. of the resultant solution, together with 0.15 ml. of 0.025 M-NaHCO₃, was added to the side arm of each manometric vessel. The enzyme, plus any salts or inhibitors, was placed in the centre compartment with sufficient 0.025 M-NaHCO₃ to make 2.7 ml. The vessels were then placed in the Warburg bath and gassed for 7 min. while shaking at 37°. During gassing some of the ethanol was driven off so that the final concentration remaining in each manometric vessel was less than 5% (v/v). This concentration of ethanol had no measurable effect on the hydrolysis of acetylcholine by human red cells and caused less than 20% inhibition with human plasma (see also O'Brien, 1956). For strict comparison the same volume of ethanol was also used in those vessels in which a choline ester was the substrate, and with all controls.

Readings were taken every 10 min., beginning 5 min. after the addition of substrate, thus allowing time for equilibration after the ethanolic substrate solution was tipped into the main compartment. When an inhibitor was used, enzyme and inhibitor were allowed to remain in contact for 20 min. at 37° before adding the substrate. Corrections for non-enzymic hydrolysis were applied in all experiments.

Under these conditions all but a slight trace of O₂ was removed from the Warburg vessels, for, in spite of the intense colour of indigo which would be formed by oxidation of enzymically liberated indoxyl with O₂, only a faint greenish coloration was produced. Thus the manometric readings could be relied upon to represent the true extent of hydrolysis. When hydrolysis was allowed to continue to completion, 80% of the theoretical yield of CO₂ was obtained from all the indoxyl substrates. This was in good agreement with the figure obtained for acetylcholine.

Owing to solubility limitations, it was not possible to determine substrate concentration-activity curves for either the indoxyl esters or α -naphthyl acetate. A substrate concentration of 6×10^{-3} M was therefore selected as being the best compromise between solubility and linearity of CO₂ production over a reasonable period. This concentration

Table 1. *Rates of hydrolysis of esters by human red cells and plasma*

Rate of hydrolysis was determined manometrically at 37° and pH 7.5 with 0.025M-NaHCO₃ as medium and a substrate concentration of 6×10^{-3} M. Enzyme activity is expressed as μ l. of CO₂/10 min./0.05 ml. of human red cells or 0.2 ml. of human plasma. All figures represent means and the standard deviation is included with each. The number of individual determinations is given in parentheses. A, Human red cells; B, human plasma.

Substrate	Choline esters	Indoxyl esters	Phenyl esters	α -Naphthyl esters
A. Acetate	70.9 $\pm$ 10.5 (23)	74.5 $\pm$ 14.9 (27)	96.7 $\pm$ 16.6 (19)	72.3 $\pm$ 5.2 (7)
Propionate	60.4 $\pm$ 12.0 (3)	29.6 $\pm$ 5.9 (8)	63.8 $\pm$ 3.6 (4)	—
Butyrate	2.0 $\pm$ 0.6 (3)	4.8 $\pm$ 2.3 (8)	6.3 $\pm$ 0.5 (3)	—
B. Acetate	102.0 $\pm$ 18.0 (26)	30.6 $\pm$ 6.0 (27)	155.9 $\pm$ 54.7 (10)	111.8 $\pm$ 16.6 (5)
Propionate	225.0 $\pm$ 19.2 (4)	70.6 $\pm$ 11.8 (7)	415 $\pm$ 86.5 (4)	—
Butyrate	313.2 $\pm$ 55.6 (4)	43.6 $\pm$ 9.4 (13)	205 $\pm$ 55.1 (4)	—

Fig. 1. Rate of enzymic hydrolysis of 6×10^{-3} M-indoxyl acetate as a function of time. O, Human red cells; ●, human plasma.

Fig. 2. Effect of increasing concentrations of eserine on the hydrolysis of acetates by human red cells. Enzyme and inhibitor were incubated together for 20 min. at 37° before adding the substrate. Substrate concentration, 6×10^{-3} M. □, Acetylcholine; ●, indoxyl acetate; ○, phenyl acetate; ■, α -naphthyl acetate.

was used in all experiments except those in which a different level is specifically mentioned. A linear output of CO₂ was maintained over a period of 1 hr., provided the enzyme concentration did not exceed that required to produce an output of 20 μ l. of CO₂/10 min. (Fig. 1). The CO₂ output during the period of linearity was proportional to enzyme concentration over a wide range, i.e. from 10 μ l. of CO₂/10 min. to 80 μ l. of CO₂/10 min.

Results quoted in Tables 1 and 2 represent the mean of a large number of determinations made on different blood samples. Individual results varied slightly from these values since, as is well known, esterase levels are not the same in all people (Augustinsson, 1955).

RESULTS

Hydrolysis by esterases of human red cells. The rate of hydrolysis of the three indoxyl esters by human red cells was measured and compared with that of the corresponding choline and phenyl esters. Table 1 shows that substrates which have the same acid radical are hydrolysed at similar rates and that α -naphthyl acetate is hydrolysed at a rate comparable with that of the other acetates. For each homologous series the rate of hydrolysis is inversely related to the length of the acyl chain.

The hydrolysis of acetylcholine by human red cells is accomplished entirely by acetylcholinesterase [as defined by its inhibition by eserine (Richter & Croft, 1942); see also Aldridge, 1954]. With indoxyl, α -naphthyl or phenyl acetate as the substrate, other esterases were found to contribute to the hydrolysis. Fig. 2 shows the effect of eserine on the hydrolysis of the four acetates. The inhibition of the hydrolysis of acetylcholine in the presence of increasing concentrations of eserine proceeds to completion in a continuous manner; this is not the case with any of the other three acetates. Eserine (10^{-6} M) inhibits the hydrolysis of indoxyl acetate by 82%; raising the inhibitor concentration to 10^{-4} M does not produce any further significant loss in enzyme activity. Similarly, the hydrolysis of α -naphthyl and phenyl acetates is not completely inhibited by 10^{-4} M-eserine.

The hydrolysis of the four acetates by human red cells is also inhibited by DFP, but its effect, like that of eserine, is always greatest with acetylcholine as substrate. A comparison of the residual activities of the same enzyme sample in the presence of 10^{-5} M-DFP and of 10^{-5} M-eserine showed that DFP consistently reduces the rate of hydrolysis of indoxyl and phenyl acetates by about 2 or 3% more than does eserine. With α -naphthyl acetate this difference is greater and amounts to 13% (Table 2).

The effect of 10^{-5} M-eserine and 10^{-5} M-DFP on the hydrolysis of indoxyl and phenyl propionates and butyrates is also shown in Table 2. As with the corresponding acetates both substances reduce the rate of enzymic hydrolysis, but the effect of DFP is always greater than that of eserine. This difference is most noticeable with the butyrates.

Table 2. *Percentage inhibition of human red-cell and human plasma enzymes by 10^{-5} M-eserine and 10^{-5} M-DFP*

Enzyme and inhibitor were incubated together for 20 min. at 37° in the centre compartment of the manometric vessel before adding the substrate from the side arm. The substrate concentration was 6×10^{-3} M. All figures represent means and the standard deviation is included with each. The number of individual determinations is given in parentheses. A, Human red cells; B, human plasma.

Substrate	Percentage inhibition							
	Choline esters		Indoxyl esters		Phenyl esters		α -Naphthyl esters	
	Eserine	DFP	Eserine	DFP	Eserine	DFP	Eserine	DFP
A. Acetate	94 ± 4.7 (3)	99 ± 1.0 (3)	83 ± 4.2 (10)	86 ± 6.0 (8)	84 ± 6.4 (14)	87 ± 5.6 (10)	70 ± 5.3 (5)	83 ± 8.5 (3)
Propionate	—	—	81 ± 5.4 (5)	93 ± 5.5 (4)	87 ± 7.7 (4)	93 ± 7.7 (3)	—	—
Butyrate	—	—	28 ± 12.1 (3)	83 ± 10.3 (3)	32 ± 5.1 (3)	97 ± 4.0 (3)	—	—
B. Acetate	100	100	90 ± 3.3 (8)	92 ± 3.5 (7)	0	0	85 ± 2.8 (4)	87 ± 2.7 (3)
Propionate	—	—	95 ± 0.2 (3)	96 ± 3.5 (3)	28 ± 11.7 (4)	31 ± 12.0 (4)	—	—
Butyrate	—	—	90 ± 5.7 (5)	92 ± 2.9 (3)	80 ± 4.3 (3)	83 ± 7.8 (3)	—	—

Table 3. *Aliphatic and aromatic esterase activities of human red cells and plasma*

Aliphatic esterase activity was determined as the difference between the activity remaining after 20 min. incubation at 37° with 10^{-5} M-eserine and that remaining after similar incubation with 10^{-5} M-DFP. Aromatic esterase activity was determined as the activity remaining after incubation of the enzyme for 20 min. at 37° with 10^{-5} M-DFP. $X = 0.02$ ml. of human red cells or 0.2 ml. of human plasma. Substrate concentration, 6×10^{-3} M.

	Esterase activity (μ l. of CO_2 /10 min.)		
	With X units of enzyme soln.	With 3X units of enzyme soln.	With 9X units of enzyme soln.
Aliphatic esterase of human red cells. Substrate: indoxyl propionate	1.5	4.4	11.5
Aromatic esterase of human red cells. Substrate: indoxyl acetate	4.1	14.3	40.2
Aromatic esterase of human plasma. Substrate: indoxyl acetate	1.1	3.4	8.5

Since 10^{-5} M-DFP inhibits acetylcholinesterase completely, the hydrolysis of the indoxyl and phenyl esters and of α -naphthyl acetate in the presence of DFP of this concentration must be due to another type of esterase. The difference in the effectiveness of eserine and DFP indicates that probably both aliphatic and aromatic esterases are involved (Mounter & Whittaker, 1953). This interpretation is supported by experiments in which higher enzyme concentrations were used. For example, the rate of hydrolysis of indoxyl acetate by red cells which had been incubated for 20 min. with 10^{-5} M-DFP is linearly related to the enzyme concentration (Table 3); control experiments with acetylcholine as substrate show that under these conditions acetylcholinesterase is completely inhibited. Table 3 also gives results for the hydrolysis of indoxyl propionate by different concentrations of red cells which had been pre-incubated for 20 min. with either 10^{-5} M-eserine or 10^{-5} M-DFP; these experiments show a linearity between the enzyme concentration and the enzymic hydrolysis which is resistant to eserine, but not affected by DFP.

That an aromatic esterase contributes to the hydrolysis of the indoxyl and phenyl esters and of α -naphthyl acetate by red cells is also shown by the effect of magnesium and *p*-chloromercuribenzoic acid on the DFP-resistant enzymic activity. Both these substances inhibit the aromatic esterase of human and rabbit plasma (Aldridge, 1953*b, c*; Hobbiger, 1954; Mounter, 1954), and the results given in Table 4 show that this also applies to the DFP-resistant hydrolysis of these substrates by red cells. Magnesium chloride (10^{-1} M) has a very similar effect on the hydrolysis of indoxyl, phenyl and α -naphthyl acetates, but the effect of 10^{-5} M-*p*-chloromercuribenzoic acid is somewhat variable. The latter could indicate that more than one aromatic esterase is involved in the hydrolysis of the different esters, but no evidence in favour of such an interpretation was obtained in experiments in which mixed substrates were used.

If several esterases contribute to the hydrolysis of a substrate their individual contributions will depend on the substrate concentration unless all the substrate concentration-activity curves are identical. Since eserine and DFP affect the enzymic hydrolysis of the indoxyl and phenyl esters in such a similar manner, phenyl acetate was taken as a model substrate for investigating the dependence of the enzymes involved on substrate concentration. The results, which are presented in graphic form in Fig. 3, show that the percentage activity of each enzyme varies with the substrate concentration. Thus the results presented in Table 2 and Fig. 2 are applicable only to the substrate concentration specified (i.e. 6×10^{-3} M).

Hydrolysis by esterases of human plasma. When human plasma is used as the source of esterases, the rate of hydrolysis is determined by both the acyl group and the rest of the substrate molecule. With the choline esters the rate of hydrolysis decreases in the following order: butyrate, propionate, acetate; with phenyl esters it is acetate, propionate, butyrate, and with indoxyl esters, propionate, butyrate, acetate (Table 1).

Pre-incubation of the plasma with 10^{-5} M-DFP for 20 min. reduces the hydrolysis of the indoxyl esters by more than 90%. The hydrolysis of α -naphthyl acetate is also greatly reduced by DFP of the same concentration, but the hydrolysis of phenyl acetate is completely unaffected. The hydrolysis of phenyl propionate and butyrate is inhibited by 31 and 83% respectively. These findings, which are

Table 4. *Inhibition of the aromatic esterases of human red cells and plasma by 10^{-1} M-magnesium chloride and 10^{-5} M-p-chloromercuribenzoic acid*

Aromatic esterase activity was determined after incubating the crude enzyme preparation with 10^{-5} M-DFP and either 10^{-1} M-MgCl₂ or 10^{-5} M-*p*-chloromercuribenzoic acid (PCMB) for 20 min. at 37° before the addition of substrate. The volume of red cells used was the same for all substrates but the volume of plasma varied by a factor of 300. Substrate concentration, 6×10^{-3} M. All figures represent the percentage inhibition in relation to controls treated with DFP only.

Substrate	Red cells		Plasma	
	MgCl ₂	PCMB	MgCl ₂	PCMB
Indoxyl acetate	67	37	66	16
α -Naphthyl acetate	64	47	97	66
Phenyl acetate	64	29	100	94
Phenyl propionate	—	—	100	89

Fig. 3. Effect of substrate concentration on the hydrolysis of phenyl acetate by the enzymes of human red cells. Curve A, total hydrolysis by red cells. Curve B, hydrolysis after incubation for 20 min. at 37° with 10^{-5} M-eserine. Curve C, hydrolysis after incubation for 20 min. at 37° with 10^{-5} M-DFP. Curve D represents hydrolysis by acetylcholinesterase (A - B); curve C represents hydrolysis by aromatic esterase; the difference between curves B and C represents hydrolysis by aliphatic esterase.

summarized in Table 2, indicate that an aromatic esterase contributes to the hydrolysis of all the esters other than those of choline. The rate of enzymic hydrolysis of these esters by DFP-treated plasma increases linearly with enzyme concentration (Table 3); control experiments with choline esters as the substrate show that under these conditions butyrylcholinesterase ('pseudocholinesterase') is completely inhibited.

Pre-incubation of the enzyme with 10^{-5} M- eserine for 20 min. has an effect which is similar to pre-incubation with 10^{-5} M-DFP (Table 2). The difference in the degree of inhibition obtained by the two inhibitors is only small and does not increase with increasing length of the acyl chain. Furthermore, to achieve a maximum inhibition by eserine it is necessary to raise the inhibitor concentration to 10^{-4} M (Fig. 4). Since no DFP-sensitive and eserine-resistant enzymic hydrolysis of the indoxyl esters is thus detectable the participation of an aliphatic esterase in the hydrolysis of these esters by plasma is unlikely.

These findings are in agreement with the conclusion of Aldridge (1953*a*) and Mounter & Whittaker (1953), that an aliphatic esterase does not participate in the hydrolysis of various phenyl esters by human plasma.

The aromatic esterase of plasma, like the aromatic esterase of red cells, is inhibited by magnesium chloride and *p*-chloromercuribenzoic acid, but the degree of inhibition achieved by a given concentration of either substance depends on the substrate used (Table 4). The great differences in the rates at which the aromatic esterase hydrolyses the various esters make it impossible to use the same enzyme concentration in all experiments. It is possible that the variation in the degree of inhibition can be at least partly attributed to the different enzyme concentrations used. Such an interpretation is supported by the finding that the hydrolysis of phenyl acetate by 1:1000 plasma is completely inhibited by 4×10^{-2} M-magnesium chloride, whereas with 1:100 plasma a concentration of more than

10^{-1} M-magnesium chloride is required to abolish aromatic esterase activity completely.

Inhibition of the aromatic esterase by *p*-chloromercuribenzoic acid enables a study to be made of the rate of hydrolysis of phenyl acetate by butyrylcholinesterase. In the absence of this substance the aromatic esterase activity is so great that human plasma can be used only in dilutions of the order of 1:1000, at which level no butyrylcholinesterase activity can be detected. In the presence of *p*-chloromercuribenzoic acid the plasma can be used

Fig. 4. Effect of increasing concentrations of eserine on the hydrolysis of acetates by human plasma. Enzyme and inhibitor were incubated together for 20 min. at 37° before adding the substrate. Substrate concentration, 6×10^{-3} M. $\square$, Acetylcholine; $\bullet$, indoxyl acetate; $\blacksquare$, α -naphthyl acetate. (Phenyl acetate is uninhibited.)

Table 5. *Relative rates of hydrolysis by purified cholinesterases*

Bovine red-cell cholinesterase and human plasma fraction IV-6-3 were used as sources of enzymes. Substrate concentration, 6×10^{-3} M. The activity is expressed as a percentage of the rate of hydrolysis of acetylcholine. Figures in parentheses represent the values for crude enzyme preparations, calculated from Tables 1 and 2. A, Acetylcholinesterase; B, butyrylcholinesterase.

	Choline esters	Indoxyl esters	Phenyl esters	α -Naphthyl esters
A. Acetate	100 (100)	70 (87)	107 (114)	53 (71)
Propionate	84 (85)	22 (34)	62 (78)	—
Butyrate	3 (3)	1 (2)	2 (3)	—
B. Acetate	100 (100)	30 (28)	54 (55)*	96 (94)
Propionate	260 (221)	72 (66)	141 (120)	—
Butyrate	332 (307)	30 (39)	190 (166)	—

* Determined in the presence of *p*-chloromercuribenzoic acid (see Results).

in a 1:100 dilution, when a large proportion of the activity is eserine-sensitive. Under these conditions butyrylcholinesterase is found to hydrolyse phenyl acetate at 55 % of the rate of acetylcholine.

Hydrolysis by purified cholinesterases. The rate at which the various substrates are hydrolysed by aceto- or butyrylcholinesterase alone is represented by the eserine-sensitive fraction of the total hydrolysis obtained with either human red cells or human plasma as source of the enzyme. An attempt was made to measure these rates directly with purified preparations of each type of cholinesterase. Bovine red-cell cholinesterase and human plasma fraction IV-6-3 were used as sources of purified enzymes.

All the esters were hydrolysed by these enzyme preparations at rates similar to those calculated from the eserine-inhibition experiments. The results are given in Table 5.

DISCUSSION

The results which have been described in this paper show that the indoxyl esters and α -naphthyl acetate, like the phenyl esters, are hydrolysed by several different enzymes. Human blood contains five different esterases which take part in the hydrolysis of these substrates, namely, acetylcholinesterase, butyrylcholinesterase, two aromatic esterases (one present in red cells and the other in plasma) and an aliphatic esterase which is found only in red cells. On the whole, the indoxyl esters and α -naphthyl acetate are hydrolysed at a far higher rate by the two cholinesterases than by the aliphatic and aromatic esterases, the hydrolysis of indoxyl butyrate by red cells being an exception.

The relative rates of hydrolysis of the indoxyl esters by acetylcholinesterase are in the descending order acetate, propionate, butyrate (Tables 1 and 2), which is the same as that previously reported by Adams (1949) for the choline esters. This confirms the previous hypothesis that there should be a biochemical similarity towards this enzyme, based upon a structural analogy between indoxyl and choline esters (Holt, 1952). On the other hand, for butyrylcholinesterase, the relative activity pattern for choline esters in descending order is butyrate, propionate, acetate (Adams & Whittaker, 1949), whereas for the indoxyl esters it is propionate, butyrate, acetate.

These findings are true for the hydrolysis of the indoxyl esters by both highly purified cholinesterases and the cholinesterases of crude enzyme preparations (Table 5). The slight differences which are observed between the rates of hydrolysis by purified and crude enzymes may be due to several factors. With acetylcholinesterase, the highly purified enzyme hydrolyses the acetates and propionates, choline esters excepted, at a slower rate than the

crude enzyme. This might be the result of a species difference between bovine and human acetylcholinesterase (Augustinsson, 1948). In both the possibility exists that other substances present in the crude enzyme may be responsible for these discrepancies (Myers, 1952).

With the aromatic esterase of red cells, a similar activity pattern to that shown by acetylcholinesterase exists for indoxyl esters, namely, acetate, propionate, butyrate. For the hydrolysis of the indoxyl esters by the aliphatic esterase of red cells and the aromatic esterase of plasma, the activity is too small to allow any definite conclusions to be drawn.

The phenyl esters are hydrolysed by the same esterases as the indoxyl esters, but there are two differences between the indoxyl and phenyl esters. First, butyrylcholinesterase has the same substrate-specificity pattern for phenyl esters as for choline esters. Secondly, the hydrolysis of the phenyl esters by the aromatic esterase of plasma proceeds at exceedingly high rates and follows the descending order acetate, propionate, butyrate. Experiments with mixed substrates have shown that the hydrolysis of both phenyl and indoxyl esters by the aromatic esterase of red cells or plasma is accomplished by the same enzyme.

Mounter & Whittaker (1953) expressed the opinion that the aromatic esterases of human red cells and human plasma were probably identical. The experiments described here show that the aromatic esterase of red cells hydrolyses indoxyl and phenyl acetates at approximately equal rates, whereas the aromatic esterase of plasma hydrolyses phenyl acetate 520 times faster than indoxyl acetate. For the propionyl and butyryl esters, this ratio is also much greater with the plasma enzyme than for the red-cell enzyme. This fact makes it very unlikely that the same DFP-insensitive enzyme is present in both red cells and plasma, even though both enzymes show similar substrate specificity and both are inhibited by magnesium chloride and *p*-chloromercuribenzoic acid.

Preliminary experiments with other tissues, such as rat liver, indicate that here non-specific esterases are present in much higher concentration than in either human red cells or plasma and that cholinesterases account for only a few per cent of the total rate of hydrolysis (Underhay, Holt, Beaufay & Duve, 1956). Unfortunately, there is no known selective inhibitor for the non-specific esterases, so that, for the time being, there is little possibility of directly studying the contribution of the cholinesterases to the hydrolysis of these substrates.

From the point of view of histochemical applications, the results obtained in this study give a strong indication that indoxyl acetates are potentially useful for the histochemical localization of the

several types of esterase, with the possible exception of the magnesium-sensitive aromatic esterases. In most cases, the localization of the total esterase activity would be demonstrated, but the use of selective inhibitors should make it possible to study directly the distribution of esterases other than the cholinesterases in tissues. In all cases, however, parallel investigations by biochemical assay of the types and activities of the esterases present in a tissue should be carried out to make possible a full assessment of the histochemical results.

Since indoxyl esters and α -naphthyl acetate are hydrolysed in a similar manner, any difference in histochemical results obtained with these two types of substrate must be associated with the kinetics of the subsequent dye-forming reaction or with the properties of the dyes formed (Holt, 1956).

SUMMARY

1. The rates of hydrolysis of three indoxyl esters (and of α -naphthyl acetate) by the esterases of human red cells and plasma have been measured and compared with those of the corresponding phenyl and choline esters.

2. All the substrates are hydrolysed by both aceto- and butyro-cholinesterase but the non-choline esters are also hydrolysed by an aliphatic and an aromatic esterase of red cells and an aromatic esterase of plasma. The contribution of the cholinesterases towards the total hydrolysis of the indoxyl esters is far greater than that of the other esterases.

3. The indoxyl and α -naphthyl esters are hydrolysed in a similar manner by the esterases, but only slowly, compared with phenyl esters, by the magnesium-sensitive aromatic esterase of plasma.

4. The aromatic esterases of red cells and plasma are not identical.

5. The relative rates of hydrolysis by the three esterases of red cells depend upon the substrate concentration employed.

6. It is concluded that the use of either indoxyl esters or α -naphthyl acetate as substrates in histochemical staining procedures leads to the demonstration of a mixture of esterases.

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Observations on the Presence of Plasma Proteins in Skin and Tendon

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It was reported in an earlier paper (Harkness, Marko, Muir & Neuberger, 1954) that a protein fraction from the skin of rabbits resembled closely serum proteins both in solubility and electrophoretic behaviour. These proteins were, however, not well

characterized and further work was considered desirable. Apart from the intrinsic interest which is attached to the proteins of connective tissue, it was hoped that a more detailed investigation of this fraction might have a bearing on the metabolism of plasma proteins. Studies by various authors, in which labelled amino acids or proteins were used, have produced fairly conclusive evidence that at

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