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The Esterases of Horse Blood

1. THE SPECIFICITY OF HORSE PLASMA CHOLINESTERASE AND ALI-ESTERASE

BY LUCY M. STURGE AND V. P. WHITTAKER
Department of Biochemistry, University of Oxford

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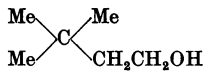
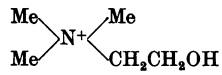
The work of Adams & Whittaker (1948, 1949), Adams (1949) and Whittaker (1949*a*) has shown that the cholinesterases of human erythrocytes, pigeon brain and human plasma are able to hydrolyse many simple aliphatic esters. For a given acyl group, the aliphatic esters most rapidly hydrolysed are those of 3:3-dimethylbutanol (I), the uncharged carbon analogue of choline (II), other esters being hydrolysed at a rate mainly dependent on the degree of their deviation from this structure. The effect of



(I)



(II)

the acyl group depends on the cholinesterase. In any series, choline or non-choline, acetate is the optimum group for the erythrocyte and brain cholinesterases, and butyrate for the plasma cholinesterase. This difference provides a basis for a nomenclature which does not depend either on the assumption that one cholinesterase is more or less specific than another or upon its supposed physiological function or lack of function. We may term all those cholinesterases possessing a specificity pattern similar to the human erythrocyte and pigeon-brain cholinesterases, acetate-cholinesterases or aceto-cholinesterases, and those possessing a specificity pattern similar to that of human plasma cholinesterase, butyrate- or butyro-cholinesterases. (The terms aceto- and butyro-cholinesterase were suggested by Dr D. Richter at the May meeting of the Biochemical Society in 1948; cf. also Augustinsson & Nachmansohn (1949), whose publication appeared during the preparation of this paper.) It is possible that other cholinesterases at present regarded as belonging to the 'true' ('specific', 'e-type') or 'pseudo' ('unspecific', 's-type') classes will prove to possess one or other of these two types of specificity pattern. To test this possibility further, the specificity of the cholinesterases of horse blood has now been investigated, the results obtained with the plasma (or serum) cholinesterase appearing in this paper, and those with the erythrocyte cholinesterase in the next (Mounter & Whittaker, 1950). We have also studied the specificity of the aliphatic esterase (ali-esterase of Richter & Croft, 1942) of horse serum with the object of defining more precisely the difference between these two types of esterase. A summary of this work has been given by Whittaker (1949*b*).

Horse serum has been a favourite source of cholinesterase for many workers, sometimes with misleading results. The native serum was found by Stedman, Stedman & Easson (1932) to hydrolyse both choline and non-choline esters, i.e. acetylcholine and butyrylcholine, methyl butyrate and tributyrin. Fractionation with $(NH_4)_2SO_4$ gave a preparation with a higher cholinesterase activity than the original serum but a lower aliphatic esterase activity. Easson & Stedman (1937) showed that suitable concentrations of prostigmine and eserine markedly inhibited cholinesterase activity while leaving the aliphatic ester hydrolysis unaffected, and also, that the rate of hydrolysis of a mixture of butyrylcholine and either methyl butyrate or tributyrin was greater than that of any of these substrates alone. They concluded that there were at least two enzymes present in horse serum, a cholinesterase and an aliphatic esterase, but admitted that the results did not exclude a small action on tributyrin by cholinesterase. Richter & Croft (1942) came to essentially the same conclusions as Stedman and his co-workers. They were able to confirm the separate identity of the aliphatic esterase (ali-esterase) by partial separation using adsorption, heat treatment or ageing and by inhibitor experiments. Glick (1938, 1939, 1941) carried out extensive specificity studies with native serum. He confirmed the increase in rate of hydrolysis found by Easson & Stedman (1936) as the homologous series of *n*-acylcholines is ascended as far as butyryl, and also showed that a further increase in acyl

group size is attended by a fall in rate of hydrolysis. He examined a few aliphatic substrates, but this part of his work is vitiated by the fact that native horse serum is now known to contain more than one esterase attacking aliphatic esters. Mendel & Rudney (1943), using various fractions of purified horse serum cholinesterase obtained by Strelitz (1944), showed that 118-fold purification was accompanied by a marked fall in the tributyrin:acetylcholine, and methyl butyrate: acetylcholine, activity ratios, but further purification did not significantly alter the residual ali-esterase: cholinesterase ratio. Eserine inhibited the cholinesterase and ali-esterase activities of their best preparations equally. They concluded that the small hydrolysis of these butyrate esters by the purified preparations was effected by the cholinesterase, which was, therefore, to this extent non-specific. Further evidence for the non-specific character of this cholinesterase comes from Augustinsson (1948) who has shown that it hydrolyses acetylaneurin, salicylcholine and acetylsalicylcholine.

In this paper we show that purified cholinesterase preparations obtained by Strelitz's (1944) method, and having the same ratio of activities towards tributyrin and acetylcholine as her preparations, are able to hydrolyse a large number of aliphatic substrates. The specificity pattern is similar to that already found for human plasma cholinesterase (Adams & Whittaker, 1949), but aliphatic esters are hydrolysed relative to acetylcholine at only about one-third to one-half the rate obtained with the human plasma enzyme, and there is a less prominent peak with esters with four carbon atoms in the acyl or alkyl group. The difference is thus not unlike that between the human-erythrocyte and pigeon-brain cholinesterases. We have also studied the specificity of the ali-esterase of horse serum. As expected, it is, in its response to changes in acyl and alkyl group size and to chain branching in the alkyl group, quite different from the cholinesterase of horse plasma; this confirms our previously expressed view that the rates of hydrolysis of simple aliphatic substrates by the cholinesterases are related to the extent to which the choline ester configuration is approached.

METHODS

Measurement of enzyme activity. Rates of hydrolysis were measured by the Warburg manometric technique (Ammon, 1933), in which the acid liberated during the course of the reaction displaces an equivalent amount of CO_2 from $NaHCO_3$-CO_2 buffer at pH 7·4 and 38°. The solution in each vessel (total vol. 3 ml.) contained 0·023 M-$NaHCO_3$ in equilibrium with 5% CO_2 + 95% N_2 in the gas phase. Aliphatic esters were added to the bottles as the pure liquid esters to give (unless otherwise stated) an 'effective' concentration (i.e. the concentration that would have been obtained if all the ester had dissolved) of approx. 1·5% (v/v). They were usually placed in the main compartment, the enzyme (adjusted to give 50–200 μl. CO_2 in 30 min.) being contained in the side bulb, but when this arrangement was reversed, and the ester added to the side bulb, as in competition or inhibitor experiments, it was covered with 0·5 ml. of 0·023 M-$NaHCO_3$. Choline esters were added as freshly prepared solutions in $NaHCO_3$ to the side bulbs to give a final concentration of 0·03 M, the enzyme being added to the main compartment. These concentrations gave constant rates of evolution for 30 min. In a few experiments with lower concentrations, no evidence was obtained of abnormal initial-velocity-concentration curves and under our conditions the enzyme appeared to be saturated.

Vessels to which the appropriate additions had been made were attached to the manometers, gassed, and equilibrated in the thermostat for 20 min. Enzyme and substrate were then mixed and after another 5 min. taps were closed and readings commenced. Initial velocities were measured in duplicate during 15 and 30 min. Non-enzymic controls were included in all experiments and the appropriate corrections made.

A few esters show troublesome negative readings (Adams, 1949) unless ample time is allowed for them to come to equilibrium with the aqueous phase. These esters were shaken with $NaHCO_3$ solution for 3 hr. and the emulsion rapidly transferred to the Warburg vessels.

Substrates. Choline esters and a number of aliphatic esters were obtained commercially; other esters were made in the laboratory by standard methods. Aliphatic esters were distilled before use, the first and last third of the distillate being rejected. They were pure as judged by the usual chemical criteria of boiling point and elementary analysis. *iso*Amyl esters were derived from technical 'amyl alcohol', containing about 90% of the *iso*-isomer. 3:3-Dimethylbutyl propionate was kindly provided by Dr A. J. Birch (see Birch, 1949, for synthesis and analysis). Abbreviations of the names of esters are given in Table 1.

Table 1. *Abbreviations of names of esters*

Acyl radical		Alcohol radical	
Ac	Acetate	Ch	Choline
Prt	Propionate	Me	Methyl
But	Butyrate	Et	Ethyl
Bz	Benzoate	Pr	Propyl
Ca	Caproate (hexanoate)	Bu	Butyl
Va	Valerate	Am	Amyl
		Hx	Hexyl

Examples. ButCh = butyrylcholine; 3:3-diMeBuBut = 3:3-dimethylbutyl butyrate.

The well known abbreviations ACh, TA and TB are retained for acetylcholine, triacetin and tributyrin.

Units. Enzymic activity is expressed in the Q notation as acid formed in μl. at N.T.P./mg. dry wt./hr. The substrate is denoted by a symbol in brackets, thus: Q(ACh), Q(TB). To conform to the conventions of the *Biochemical Journal* (see *Suggestions to Authors*, 1949) the acid formed should appear as a subscript thus: $Q_{\text{acetic acid}}$(ACh), $Q_{\text{butyric acid}}$(TB). To save space these subscripts have been omitted throughout. Dry weights were determined by drying 1 ml. of dialysed solution at 110°.

Rates are also frequently expressed as percentages of the acetylcholine rate under identical conditions of enzyme concentration, temperature and pH, duplicate determinations agreeing to within 5%. Degrees of purification are the ratios of the Q values of purified fractions to those of the starting materials. Yields are obtained by expressing the total activity of a final fraction (μl. acid (or CO_2)/ml./hr. × vol. of fraction in ml.) as a percentage of the initial total activity.

RESULTS

A. Cholinesterase

Preparation of cholinesterase

Cholinesterase was prepared from sterile citrated horse plasma and horse serum provided by the Experimental Station, Porton, and stored at 0° after the addition of a few drops of chloroform. The first three stages of Strelitz's (1944) purification were used as a basis. The object of purification was primarily to obtain an enzymically homogeneous cholinesterase preparation free from ali-esterase, and only secondarily to obtain a highly active one. Triacetin was used to measure the ali-esterase activity; the rate of hydrolysis of this substrate by native plasma or serum is very variable, sometimes being as much as three times that of acetylcholine. Even a moderate degree of purification greatly reduces the rate of hydrolysis of triacetin relative to that of acetylcholine, but we have not been able to reduce it below about 6 % of the acetylcholine rate (see Table 2), and it is likely that this represents the contribution of the cholinesterase to the total triacetin hydrolysis. We have therefore used a triacetin hydrolysis of this figure as a working criterion of freedom from ali-esterase.

With serum, no difficulty was experienced in repeating Strelitz's method, but with citrated plasma certain discrepancies were noted after her stage 1. Thus, in the second stage no precipitate was obtained when the solution was 0·4 saturated with ammonium sulphate; this stage was therefore omitted and the solution brought to 0·45 saturation and pH 3·5 (stage 3). This treatment precipitated most of the cholinesterase instead of leaving it in solution.

Typical preparations were as follows:

(1) Plasma (1 l.; Q(ACh), 56; Q(TA), 44) was taken through stage 1. A precipitate (1a), obtained by 70 % saturation with $(NH_4)_2SO_4$, was taken up in water and dialysed. Purification 13-fold, yield 46 %.

(2) Plasma (1 l.; Q(ACh), 27; Q(TA), 84). A precipitate, obtained as in preparation 1, was extracted with 20 ml. of acetate buffer pH 3·5. The extract after 45 % saturation with $(NH_4)_2SO_4$ yielded a precipitate (2a) which contained most of the activity. This was dissolved in water and dialysed. Purification 70-fold, yield 25 %.

(3) Serum (1 l.; Q(ACh), 35; Q(TA), 104) was taken to stage 3 of Strelitz's preparation. After dialysis the clear filtrate (3a) had purification 110-fold, yield 10 %.

No difference was detected in preparations derived from plasma and serum and these terms are used interchangeably to designate the source of the enzyme.

Evidence of homogeneity

Effect of increasing purification. Table 2 shows that further purification did not eliminate the hydrolysis of aliphatic esters shown by partially purified cholinesterase, and left the ratios of activities unaffected. The figures for tributyrin are approximately equal to (actually a little lower than) those obtained by Mendel & Rudney (1943) and Strelitz (1944) in preparations up to 600 times as active as the original serum. There is little doubt, therefore, that all these fractions were enzymically homogeneous and did not contain ali-esterase. That the enzyme removed in the purification was an ali-esterase and not a second cholinesterase is supported by the fact that the benzoylcholine : acetylcholine ratio, unlike the aliphatic ester : acetylcholine ratios, was unaffected by purification.

Inhibitor experiments. Experiments with eserine confirmed that the hydrolysis of aliphatic substrates by these purified fractions was effected by the cholinesterase. Table 3 shows that 10^{-5}M-eserine, a concentration shown by Richter & Croft (1942) to inhibit horse serum cholinesterase completely without affecting the ali-esterase, produced complete or almost complete inhibition of the hydrolysis of both choline and non-choline esters by one of our purified fractions.

Competition experiments. Table 4 shows that choline and non-choline esters compete for the horse plasma cholinesterase. It will be noted that the rate of hydrolysis of the mixed esters lay, with each pair of esters, between the rates of hydrolysis of each ester measured separately. If the hydrolysis of the aliphatic ester had been due partly or wholly to a

Table 2. *Effect of purification on the specificity of horse-plasma cholinesterase*

Preparation	Q(ACh)	Degree of purification	Rate of hydrolysis of other substrates expressed as percentage of ACh rate				
			TA	TB	*iso*AmAc	*iso*AmBut	BzCh
			Unpurified material				
1	56	—	78	—	—	—	—
2	27	—	313	198	578	235	31
3	35	—	297	—	—	—	—
			Purified fractions				
1a	725	13-fold	6·4	30	7·8	13·3	31
2a	1900	70-fold	6·3	24	7·7	13·3	—
3a	4150	110-fold	5·9	28	8·5	13·5	29

second enzyme we should have expected the rate of hydrolysis of the mixed esters to have exceeded that of either ester alone. This state of affairs is indeed shown in the last line of Table 4, which records an experiment with a preparation of ali-esterase containing some cholinesterase. It will be seen that the rate of hydrolysis of the mixed esters exceeded that of either ester alone, but was not quite equal to the sum of their rates. As the contribution of the cholinesterase to the hydrolysis of the aliphatic substrate could only have been 2 or 3 μl., it is possible that butyrylcholine was weakly inhibiting the ali-esterase.

Table 3. *Inhibition of ester hydrolysis by eserine*

(Preparation 3*a* (see Table 2); choline esters, 30 mM; aliphatic esters, 100 mM; eserine, 10 μM.)

Substrate	Inhibition (%)
ACh	99
BzCh	89
TA	100
TB	100
2-EtBuAc	100
n-AmPrt	100
*iso*AmPrt	100
*iso*AmBut	99

The specificity of the plasma cholinesterase

As in earlier studies (Adams & Whittaker, 1949), the specificity of preparations of purified cholinesterase has been explored by measuring the rates of hydrolysis of a large number of aliphatic substrates; these are expressed as a percentage of the rate of hydrolysis of acetylcholine under identical conditions. The preparations used were those listed in the previous section and fractions of another preparation similar to no. 2.

The effect of increasing chain length in the acyl radical of homologous series of aliphatic esters is shown in Fig. 1*a*, and in the alkyl radical in Fig. 2*a*. A comparison with Adams & Whittaker's data (reproduced in Figs. 1*b* and 2*b*) shows at once that while the general pattern of activity of the two enzymes is similar, the aliphatic esters are split at a half or less of the rate relative to acetylcholine found with the enzyme of human origin, and the sharp peak with butyl and with butyrate found with the human enzyme is not obtained here. The effect of chain

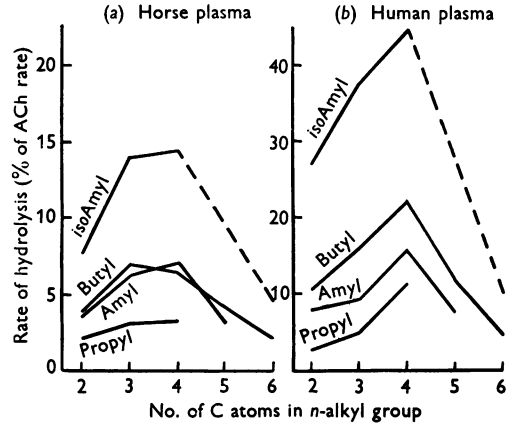


Fig. 1. Effect of changes in acyl group size on rate of hydrolysis of aliphatic esters by plasma cholinesterase. (*a*) Horse plasma. (*b*) Human plasma.

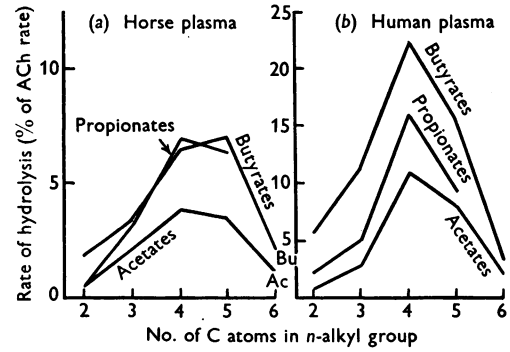


Fig. 2. Effect of changes in alkyl group size on rate of hydrolysis of aliphatic esters by plasma cholinesterase. (*a*) Horse plasma. (*b*) Human plasma.

branching in the alkyl group on the rate of hydrolysis of one series of aliphatic esters (propionates) is shown in Fig. 5*a*. Again the branched-chain esters are hydrolysed somewhat less rapidly than was to be expected from the results with the human enzyme,

Table 4. *Competition for horse-plasma esterases by choline and non-choline esters*

Substrates		Rate of hydrolysis (μl. CO_2/0·1 ml./hr.) of			
(*a*)	(*b*)	(*a*)	(*b*)	Mixed (*c*)	(*c* − *a*)
		Cholinesterase			
BzCh	*iso*AmAc	440	131	314	−126
BzCh	*iso*AmBut	411	180	390	− 21
BzCh	*iso*AmCa	365	49	364	− 1
BzCh	TB	234	206	108	−126
		Ali-esterase			
*iso*AmAc	ButCh	253	77	277	+ 24

but again there is the same pattern of specificity, and the same marked increase in rate as the choline configuration is approached which seems to be characteristic of cholinesterases. Data for three additional compounds not shown in the graphs are given in Table 5.

Table 5. *Rates of hydrolysis of butyrylcholine, benzoylcholine and 2-ethylbutyl acetate by horse-plasma cholinesterase*

Substrate	Rate of hydrolysis (% of ACh rate)
ButCh	250
BzCh	29
2-EtBuAc	2

B. Ali-esterase

The specificity of the ali-esterase of horse plasma was studied by three methods.

(1) By measuring the rate of hydrolysis of aliphatic esters by native plasma with suitable correction for the contribution made by the cholinesterase. This was obtained from the acetylcholine rate and the percentage rate of hydrolysis of the aliphatic substrate determined during the investigation of the specificity of the cholinesterase. Most determinations were carried out on plasma 2 which had a particularly high ali-esterase activity. For most substrates, the correction was less than 10%, and the highest (for tributyrin and 3:3-dimethylbutyl propionate) was only about 15%. As an example, the rate of hydrolysis of *iso*amyl acetate by native plasma was 10,170 μl. CO_2/ml./hr.; that of acetylcholine was 2030. Cholinesterase hydrolyses *iso*amyl acetate at 7·8% of the acetylcholine rate and so contributed $2030 \times 0{\cdot}078$ or 160 μl./ml./hr. to the total hydrolysis. Ali-esterase must therefore have contributed 10,170 − 160 or 10,010 μl./ml./hr.

(2) Some measurements were made in the presence of 10^{-5} M-eserine, which completely, or almost completely, abolishes cholinesterase activity.

(3) Partial separation of ali-esterase from cholinesterase was effected by fractionation with ammonium sulphate.

Partial separation of ali-esterase from cholinesterase. Experiments in which native plasma was submitted to stepwise salting out with $(NH_4)_2SO_4$, and observations made during the cholinesterase preparations, suggested that the ali-esterase was mainly precipitated with the albumin fraction. Accordingly, an attempt was made to separate ali-esterase by conforming to conditions which favour a sharp separation of albumin, i.e. the use of fresh serum and precipitation by 0·5 saturation at pH 4·6 after removal of globulin. Serum (1 l.) was diluted with glass-distilled water (350 ml.) and brought to 0·5 saturation by the addition of 1350 ml. of saturated $(NH_4)_2SO_4$ at 0° during 1 hr. After 3 hr. the serum globulin was removed by vacuum filtration through an 18·5 cm. Whatman no. 54 filter paper. The filtrate was brought to pH 4·6 with glacial acetic acid, allowed to stand 10 hr. and refiltered as before. The light-yellow precipitate was taken up in water and neutralized with N-NaOH, dialysed and $NaHCO_3$ added to 0·2% (w/v).

Table 6 shows that, although the *Q*(*iso*AmAc) of the albumin fraction had been somewhat reduced (to about 80% of the initial value), the *Q*(ACh) value was only about one-quarter of the original value and the *Q*(*iso*AmAc)/*Q*(ACh) ratio had been trebled. The cholinesterase content is so low that its contribution to ali-esterase hydrolysis for most substrates is negligible.

Table 6. *Separation of ali-esterase and cholinesterase activities*

Fraction	*Q*(ACh)	*Q*(*iso*AmAc)	*Q*(*iso*AmAc)/*Q*(ACh)
Original serum	39	158	4·1
Albumin fraction	10	121	12·4

Recovery of ali-esterase activity in the various fractions of the cholinesterase preparations was low when plasma was used as the source of enzyme, but was better (60%) with serum. It is possible that the 'albumin' fraction from a serum preparation would be a suitable starting material for further work.

Comparison of different methods. In Table 7 are presented specificity data obtained by the three methods described above. Taking the rate of hydrolysis of *iso*AmAc as standard and expressing the rates of hydrolysis of the other aliphatic substrates as a percentage of this rate, good agreement (approx. 10% or less) is obtained between the different methods, except for three substrates, PrBut, TA and

Table 7. *Comparison of specificity data for horse-serum ali-esterase obtained by three methods*

(Rate of hydrolysis is expressed as percentage of rate of hydrolysis of *iso*amyl acetate. (*a*) Rate obtained with unpurified plasma corrected for cholinesterase. (*b*) Rate obtained with unpurified plasma in presence of 10 μM-eserine. (*c*) Rate obtained with partially purified preparation corrected for residual cholinesterase. No. of determinations averaged is shown in brackets.)

Substrate	Rates of hydrolysis (*a*)	(*b*)	(*c*)
*iso*AmAc	100	100	100
*iso*AmPrt	73 (3)	71	76 (2)
*iso*AmBut	46 (3)	45	47 (2)
AmAc	66 (2)	—	71
AmPrt	49 (2)	—	55 (2)
PrPrt	45 (2)	—	53
PrBut	72 (2)	73	93 (2)
BuPrt	92 (2)	—	108 (2)
BuBut	71 (2)	69	74
TA	62 (4)	59 (2)	87 (3)
TB	35 (2)	33	49
2-EtBuAc	146 (2)	—	147
MeBut	52 (2)	57	—
3:3-diMeBuPrt	37 (2)	37	—
BzCh	0	0	—

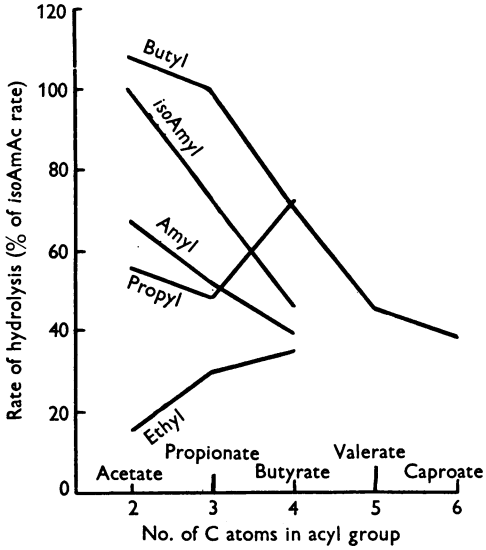


Fig. 3. Effect of changes in acyl group size on relative rates of hydrolysis of aliphatic esters by horse plasma ali-esterase.

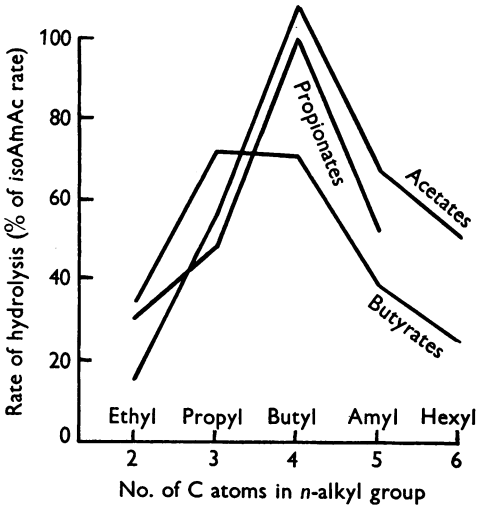


Fig. 4. Effect of changes in alkyl group size on relative rate of hydrolysis of aliphatic esters by horse plasma ali-esterase.

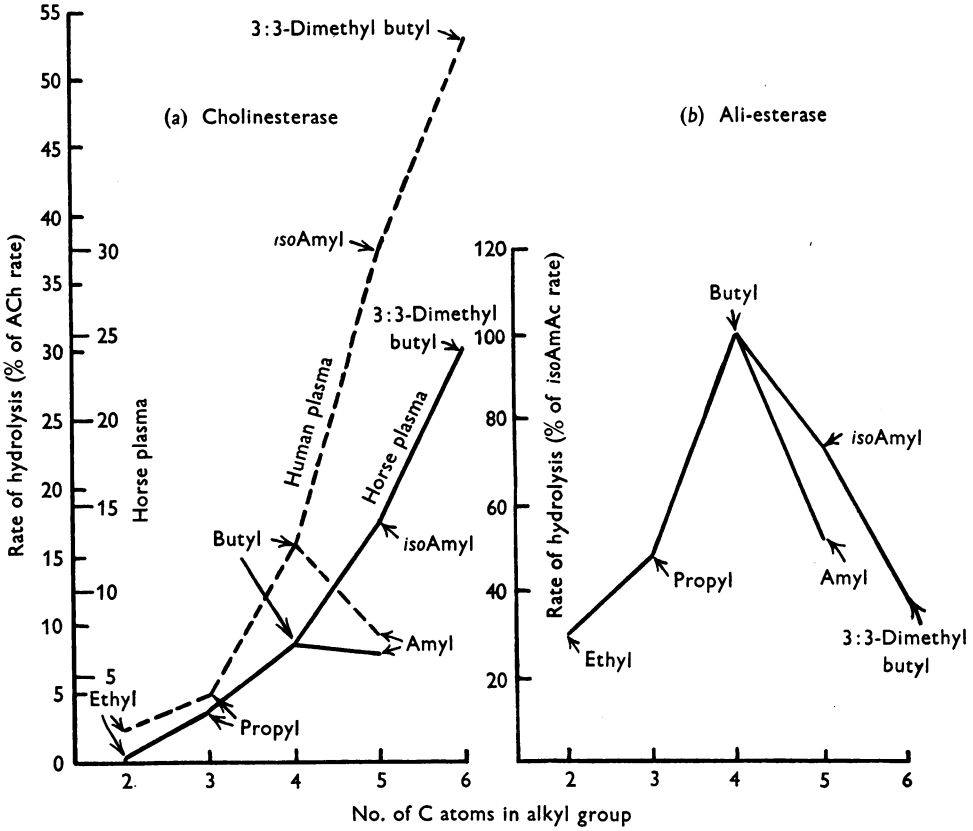


Fig. 5. Effect of chain branching in alkyl group on rate of hydrolysis of propionate esters by (*a*) human and horse plasma cholinesterase, (*b*) horse plasma ali-esterase. The outer scale of (*a*) refers to human plasma and the inner scale to horse plasma.

TB. Although the rates of hydrolysis of these substrates in the presence of eserine agree well with those obtained with native plasma by correcting for cholinesterase, the rates obtained with the partially purified ali-esterase are distinctly higher. This will be commented on later. In the graphs these high rates have been neglected when taking an average. The failure of the ali-esterase to attack benzoylcholine should also be noted.

Specificity of ali-esterase. The data are presented graphically in Figs. 3, 4 and 5*b* in such a way as to illustrate the effect of change of acyl group size (Fig. 3), alkyl group size (Fig. 4) and chain branching (Fig. 5*b*) on the rate of hydrolysis. Comparison with the data for cholinesterase (Figs. 1*a*, 2*a* and 5*a*) shows that an entirely different specificity pattern is obtained. In Fig. 3 it will be seen that increasing acyl group size markedly decreases, in three series of esters, the rate of hydrolysis by ali-esterase. The ethyl esters, the least rapidly hydrolysed, behave, however, in the opposite way. The propyl series shows an intermediate behaviour in that an increase from acetate to propionate leads to a fall in the rate of hydrolysis, but with the butyrate there is a marked increase, which exceeds the preceding fall.

In Fig. 4, it will be seen that there is a tendency for a peak to occur at *n*-butyl, but again the rate of hydrolysis of propyl butyrate prevents a satisfactory generalization being made. A comparison of Fig. 5*a* with Fig. 5*b* shows that the effect of chain branching is quite different in the two enzymes. Although with both the butyl ester is the most rapidly hydrolysed of the *n*-alkyl propionates, the peak is actually much more pronounced with the ali-esterase. But whereas chain branching in the 3-position as in primary *iso*-amyl propionate, 3:3-dimethylbutyl propionate, is accompanied by a big increase in rate of hydrolysis by the cholinesterase, it causes a fall in the rate of hydrolysis by the ali-esterase. The two enzymes also respond differently to chain branching in the 2-position. Substitution of an ethyl group in the 2-position in butyl acetate causes a fall in the relative rate of hydrolysis by cholinesterase of nearly 50 %, but a rise in the rate of hydrolysis by the ali-esterase of 35 %.

DISCUSSION

The close similarity between human and horse plasma which has been generally accepted by previous workers extends to their behaviour towards non-choline esters. Like the human enzyme, the horse enzyme hydrolyses aliphatic esters at a rate which increases with the extent to which they approach the configuration of choline esters; also like the human enzyme, it hydrolyses butyryl esters more rapidly than acetates in every series so far investigated, whether choline, glyceryl or alkyl. In the four series for which there are data, the choline (Glick, 1941), butyl, *iso*amyl and amyl series, an increase in acyl group size beyond butyryl is attended by a fall in the rate of hydrolysis. It is clear, therefore, that the butyrate group is the optimum acyl group for the horse as well as for the human plasma cholinesterase; this justifies the classification of the former enzyme along with the latter as a butyrocholinesterase. There are, however, certain differences in detail between the two enzymes. Thus the rate of hydrolysis of both glyceryl and simple aliphatic esters is invariably slower, relative to acetylcholine, with the horse than with the human enzyme; this may be of significance in relation to the much higher ali-esterase activity content of horse plasma. Further, the peak with butyl propionate is less marked, butyl butyrate, *n*-amyl propionate and *n*-amyl butyrate being split at almost the same rate. These two differences may possibly be related: we should expect structural changes to have relatively less effect in substrates which as a class are not so rapidly hydrolysed.

A possible, though unlikely, explanation of the alkyl specificity pattern of the cholinesterases is that the varying rates of hydrolysis of the aliphatic esters are not primarily determined by the nearness of approach to the choline configuration, but by varying degrees of chemical activation of the ester link by the variously branched and substituted alkyl chains. Although, no doubt, chemical effects may contribute to, and on occasion overshadow, the configurational factor, they cannot be dominant in the series of simple aliphatic esters used here, or we should expect to find essentially the same specificity pattern with esterases which do not hydrolyse choline esters. An esterase of this type, the ali-esterase of horse plasma, showed, in fact, a completely different pattern from the cholinesterase, in spite of the close association and somewhat similar physical properties of these two enzymes. The same argument also excludes solubility as a determining factor. The specificity pattern of the ali-esterase, unlike that of the cholinesterase, does not, however, lend itself to any simple interpretation, though it is possible that tests with a wider selection of esters would reveal one. Our main object has, however, been to find how an aliphatic esterase would respond to esters the configuration of which could be related to that of choline.

The ester most rapidly hydrolysed by the ali-esterase is 2-ethylbutyl acetate (nearly 150 % of *iso*-AmAc rate), but all the esters tested (except ethyl esters) gave values between about 35 and 110 % of this rate. The two glyceryl esters tested fall in the lower half of this range and there is certainly no evidence for a preference for a glyceryl configuration. Acetate appears to be the optimum acyl group for most series of esters and a four-carbon chain in the alcohol group also favours a high rate of hydrolysis. Propyl butyrate and, to a lesser extent, the ethyl

esters are exceptions. It is possible that the butyrate group is to some extent interchangeable with butyl, i.e. $R.O.CO.CH_2CH_2CH_3$ is equivalent to $RCO.O.CH_2CH_2CH_2CH_3$, but a more likely explanation is that the ali-esterase preparations are not homogeneous and that they contain a small quantity of a second ali-esterase with a specificity pattern the reverse of the one postulated above, i.e. an enzyme which hydrolyses butyrates more rapidly than acetates and ethyl esters $\geqslant$ propyl $\gg$ other esters. The composite pattern could be similar to the one actually obtained. It is possibly significant, therefore, that in Table 7, the rate of hydrolysis of propyl butyrate by the preparation which has been partially fractionated (a procedure which might tend to alter the ratio of a mixture of enzymes) does not agree with those obtained from unpurified preparations, whereas the agreement for all the other simple esters tested is excellent. The only other discrepancies are with the two glyceryl esters, which would be expected if both ali-esterases hydrolyse glyceryl esters, but at different rates. Further fractionation of the plasma ali-esterase will clearly be necessary before these results can be fully explained.

SUMMARY

1. The cholinesterase of horse plasma has been found to resemble human plasma in hydrolysing a large number of aliphatic esters. The results are consistent with the hypothesis that the nearer the configuration of butyrylcholine is attained the greater the rate of hydrolysis.

2. A possible alternative view, that the variation in the enzymic rates of hydrolysis of aliphatic esters is due merely to changes in chemical activation associated with differing substitution and chain branching, would seem to imply that all esterases, whether able to hydrolyse choline esters or not, would show essentially the same specificity pattern with aliphatic esters. This view is rendered unlikely, and our own interpretation confirmed, by the finding that the ali-esterase and cholinesterase of horse plasma, in spite of their close association and physical similarity, have quite distinct specificity patterns.

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The Esterases of Horse Blood

2. THE SPECIFICITY OF HORSE ERYTHROCYTE CHOLINESTERASE

By L. A. MOUNTER and V. P. WHITTAKER
Department of Biochemistry, University of Oxford

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A brief account of previous work by one of us (V. P. W.) and D. H. Adams on the specificity of the cholinesterases of human blood and pigeon brain has been given in the preceding paper (Sturge & Whittaker, 1950). The results obtained have made desirable further investigations designed to reveal to what extent the specificity patterns of the cholinesterases of different species conform to the two main types so far discovered.

Little previous study has been made of the specificity of horse erythrocyte cholinesterase, perhaps because horse erythrocytes are not a good source of the enzyme, having only about one-fifth of the activity of human erythrocytes. Mendel & Rudney (1943) and Mendel, Mundell & Rudney (1943) showed that the partially purified enzyme free from ali-esterase, like the human erythrocyte enzyme, hydrolysed, in addition to acetylcholine, acetyl-β-