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.

We would like to express our thanks to Dr D. R. Davies, Porton, for supplies of horse plasma and serum and to the Medical Research Council for grants.

REFERENCES

Adams, D. H. (1949). *Biochim. Biophys. Acta*, **3**, 1.
Adams, D. H. & Whittaker, V. P. (1948). *Biochem. J.* **43**, xiv.
Adams, D. H. & Whittaker, V. P. (1949). *Biochim. Biophys. Acta*, **3**, 358.
Ammon, R. (1933). *Pflüg. Arch. ges. Physiol.* **233**, 486.
Augustinsson, K. B. (1948). *Acta physiol. Scand.* **15**, suppl. 52.
Augustinsson, K. B. & Nachmansohn, D. (1949). *Science*, **110**, 98.
Birch, A. J. (1949). *J. chem. Soc.* p. 2721.
Easson, L. H. & Stedman, E. (1936). *Proc. roy. Soc.* B, **121**, 142.
Easson, L. H. & Stedman, E. (1937). *Biochem. J.* **31**, 1723.
Glick, D. (1938). *J. biol. Chem.* **125**, 729.
Glick, D. (1939). *J. biol. Chem.* **130**, 527.
Glick, D. (1941). *J. biol. Chem.* **137**, 357.
Mendel, B. & Rudney, H. (1943). *Biochem. J.* **37**, 59.
Mounter, L. A. & Whittaker, V. P. (1950). *Biochem. J.* **47**, 525.
Richter, D. & Croft, P. G. (1942). *Biochem. J.* **36**, 746.
Stedman, E., Stedman, E. & Easson, L. H. (1932). *Biochem. J.* **26**, 2056.
Strelitz, F. (1944). *Biochem. J.* **38**, 86.
Whittaker, V. P. (1949*a*). *Biochem. J.* **44**, xlvi.
Whittaker, V. P. (1949*b*). *1st Int. Congr. Biochem. Abstr.* p. 587.

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

(*Received* 3 *August* 1949)

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-

methylcholine, $[Me_3N^+.CH_2CHMe.OAc]OH^-$, but not benzoylcholine or tributyrin. Augustinsson (1948) found no hydrolysis of *N*-acetyl-*p*-aminobenzoylcholine and carbaminoylcholine, but his data for certain other esters which he states are hydrolysed by cholinesterase must be taken with reserve, as his preparations contained considerable quantities of ali-esterase.

In this paper we show that the specificity of horse erythrocyte cholinesterase, carefully freed from ali-esterase, is almost identical with that of the human erythrocyte enzyme, a wide range of simple aliphatic esters being hydrolysed at a rate which can be correlated with the extent to which they approach the configuration of acetylcholine. This close similarity is interesting in view of the appreciable difference in detail between the properties of human and horse plasma cholinesterases, but final proof of the identity of the two enzymes must await their isolation in a pure state.

METHODS

Substrates. The substrates were those described in the previous paper, with the addition of propionylcholine (made from choline perchlorate), acetyl-β-methylcholine, benzyl acetate (both obtained commercially) and 3:3-dimethylbutyl acetate (kindly provided by Dr A. J. Birch; see Birch, 1949, for preparation and analysis). Aliphatic substrates were distilled before use and satisfied the usual chemical criteria of purity. They were used as before in an 'effective' concentration of 0·1 M, acetyl-β-methylcholine in 0·03 M concentration and acetylcholine 0·006 M (0·03 M produces marked inhibition by excess substrate). As in previous work (Adams & Whittaker, 1948; Adams, 1949; Whittaker, 1949), acetyl-β-methylcholine was used as a standard substrate, because, unlike acetylcholine, it has a normal initial velocity-substrate concentration curve and does not show inhibition by excess substrate. Abbreviations of the names of esters are given in Table 1.

Table 1. *Abbreviations*

Ac	Acetate	Ch	Choline	Bu	Butyl
Prt	Propionate	Me	Methyl	Am	Amyl
But	Butyrate	Et	Ethyl	Hx	Hexyl
		Pr	Propyl	Bzl	Benzyl

ACh, Acetylcholine; TA, Triacetin; TB, Tributyrin; MCh, Acetyl-β-methylcholine.

Estimation of enzyme activity. The methods used to determine enzyme activity, *Q* values, degrees of purification and yields were essentially as described in the previous paper (Sturge & Whittaker, 1950), except that as measurements had sometimes to be made on solutions containing salts, dry weights were determined by heating samples with 5% (w/v) trichloroacetic acid for 10 min. in boiling water, collecting the coagulated protein on a tared G4 sintered-glass filter, washing, and drying to constant weight. Activities are expressed as μl. CO_2/ml./30 min., as *Q* values (μl. acid/mg. dry wt./hr.), and as a percentage of the activity obtained with acetyl-β-methyl-choline under identical conditions.

RESULTS

Partial purification of horse erythrocyte cholinesterase

In studying the ability of cholinesterases to hydrolyse non-choline esters, it is essential to remove the ali-esterase which usually accompanies them. There is a considerable amount of tributyrinase in the horse erythrocyte. By using an improved method of purification described below we have been able to reduce the tributyrin hydrolysis from about 50 to 1% or less of the acetyl-β-methylcholine hydrolysis, and we have used the tributyrin hydrolysis as a routine test for the presence of ali-esterase in our preparations. A method of purification differing but slightly from that of Mendel & Rudney (1943) and involving adsorption of the enzyme on kieselguhr, which had proved satisfactory with human erythrocyte cholinesterase (for details see Adams, 1949), gave with horse erythrocytes a purification of only 13-fold with an 11% yield, possibly because of their low cholinesterase content. Paléus (1947) and Augustinsson (1948) have found that erythrocyte cholinesterase is bound to the cell membrane and so can be precipitated along with the stromata at about pH 6·5. Some figures of Augustinsson for horse erythrocytes show, however, that precipitation reduces, but by no means eliminates, the erythrocyte tributyrinase. Table 2, which shows the relevant data from

Table 2. *Precipitation of horse erythrocyte esterases with stromata*

(Substrates, 0·25% (w/v); data of Augustinsson, 1948.)

	Activity (μl. CO_2/30 min.)		
	ACh	TB	TB/ACh (%)
Supernatant + ppt., pH 6·75	50	44	88
Precipitate, pH 6·75	42	14	33

Table 13 of his monograph, demonstrates that although acidification to pH 6·75 precipitated only 32% of the tributyrin activity as against 84% of the acetylcholine activity, the tributyrin: acetylcholine ratio was still high (33%) in the precipitated material. Nevertheless, it seemed worth while to attempt to combine the precipitation and adsorption techniques, making use of precipitation to effect a preliminary concentration of the enzyme and the adsorption method to remove the remaining tributyrinase. Preliminary studies showed that precipitation was maximal at pH 6·3–6·5 with our erythrocytes, and that redispersion occurred when the solution was neutralized, being complete at pH 8; also that a weakly acid pH is needed for maximum adsorption on kieselguhr and that elution could be effected by dilute sodium hydroxide or bicarbonate at pH 8. Furthermore, freshly laked erythrocytes

are essential for a successful separation of tributyrinase; the age of the erythrocytes before laking is, however, immaterial.

The final method of purification was as follows. Packed horse erythrocytes (Burroughs Wellcome Ltd., stored at 0°) (500 ml.) having $Q(MCh)=2{\cdot}7$, $Q(TB)=1{\cdot}1$ were laked by the addition of 1·5 l. of water. The pH was reduced to 6·5 with 0·1 N-HCl (pH was measured with a glass electrode) and the stromata were removed by centrifuging. After washing with two 100 ml. portions of water, they were resuspended in 200 ml. of water and the solution brought to pH 8 with 0·1 N-NaOH. Kieselguhr (35 g.) was added and the pH reduced slowly with shaking to 6·3 with 0·1 N-HCl. The kieselguhr was collected by filtration, washed with two portions of 100 ml. of water and the enzyme eluted with 150 ml. of 0·2% $NaHCO_3$. The eluate, which contained the enzyme as a fine dispersion, was freed from kieselguhr by centrifuging at 500 *g.* for 10 min. It was then centrifuged at about 3500 *g.* for 2 hr. and the precipitated enzyme, after being washed with 0·2% $NaHCO_3$ and recentrifuged, was resuspended in 50 ml. of $NaHCO_3$. Purification, 23-fold; yield, 46%. The enzyme, when stored at 0° with a few drops of $CHCl_3$ as a preservative, was stable for several weeks.

After the completion of this work we encountered a paper by Scheiner (1948) in which a purification procedure similar to our own is described. Scheiner, however, gives few practical details and does not state whether his product was free from ali-esterase.

Criteria of homogeneity

Adams (1949) has given a full account of methods which confirm the homogeneity of preparations of human erythrocyte cholinesterase with which the tributyrin hydrolysis is less than 2·5% of the acetyl-β-methylcholine hydrolysis. As the relative tributyrin hydrolysis by our preparations of horse erythrocytes was never greater than 1%, it may be assumed that they contained a negligible quantity of ali-esterase. The reduction of the tributyrinase activity as a result of purification is illustrated in Table 3. In Table 4 it is seen that an aliphatic ester,

Table 3. *Cholinesterase and tributyrinase activity of laked horse erythrocytes and purified preparations*

(MCh, 30 mM; TB, 100 mM; activity units, μl. CO_2/ml./30 min.)

Laked erythrocytes			Purified preparation		
MCh	TB	TB/MCh (%)	MCh	TB	TB/MCh (%)
246	125	52	516	2	0·5
252	116	46	248	3	1

*iso*amyl acetate, competes with acetyl-β-methylcholine for the purified cholinesterase, the rate of hydrolysis of the mixed esters lying between that of acetyl-β-methylcholine and that of the aliphatic ester. If the latter had been hydrolysed by a second enzyme, we should expect the rate of hydrolysis of the mixed esters to lie above the rate of either substrate alone. Table 5 compares the effects of 10^{-5} M-eserine (cf. Richter & Croft, 1942) on the hydrolysis of choline and non-choline esters by the original laked erythrocytes and a purified preparation. It

Table 4. *Competition of a choline and non-choline ester for purified horse erythrocyte cholinesterase*

(MCh, 30 mM; *iso*AmAc, 100 mM; activity units, μl. CO_2/ml./30 min.)

MCh	*iso*AmAc	Mixed
516	364	476
516	352	472

Table 5. *Inhibition of choline and non-choline ester hydrolysis by eserine*

(MCh, 30 mM; TB and AmAc, 100 mM; eserine, 10 μM. Activity units, μl. CO_2/ml./30 min. Eserine was incubated with the enzyme in the absence of substrate for 10 min. at 38°.)

Laked erythrocytes		Purified preparation	
Control	Inhibited	Control	Inhibited
	MCh		
244	0	516	4
248	0	512	0
	TB		
124	118	0	1
126	122	4	2
	AmAc		
220	133	198	3
222	130	210	1

will be seen that this concentration of inhibitor produces complete, or almost complete, inhibition of acetyl-β-methylcholine hydrolysis by both preparations. It produces only slight inhibition of the hydrolysis of tributyrin by unpurified erythrocytes; the tributyrin hydrolysis by the purified preparation is too low to be measured accurately in the presence or absence of eserine in spite of the fact that the

Table 6. *Constancy of specificity in different preparations of horse erythrocyte cholinesterase*

(Enzyme activity in μl. CO_2/ml./30 min.; figures in brackets give rates as percentage of MCh rate. Each line corresponds to a different preparation.)

MCh	*iso*AmAc	*iso*AmPrt	PrAc	EtAc	HxAc
203	—	—	—	—	55 (27)
248	180 (72)	69 (28)	72 (29)	10 (4)	—
516	372 (72)	144 (28)	118 (31)	18 (4)	126 (25)

enzyme concentration was increased fivefold in these experiments. *n*-Amyl acetate is hydrolysed nearly as rapidly as acetyl-β-methylcholine by unpurified erythrocytes; the inhibition by eserine, though greater than that with tributyrin as a substrate, is by no means complete. Amyl acetate hydrolysis is not eliminated, as tributyrin hydrolysis is, by purification

(although it is reduced somewhat relative to acetyl-β-methylcholine) and the remaining hydrolysis is now almost completely eserine-sensitive. These facts, together with the constant specificity of different preparations (Table 6), confirm that purification, although eliminating ali-esterase, does not abolish the hydrolysis of all aliphatic esters, and show that this aliphatic esterase activity must be due, with the horse as well as with the human erythrocyte, to the cholinesterase.

Specificity

The rates of hydrolysis by the horse erythrocyte cholinesterase of a number of aliphatic esters, expressed as a percentage of the rate of hydrolysis of acetyl-β-methylcholine under identical conditions of enzyme concentration, pH and temperature, are presented in Figs. 1 and 2 and Table 7. It will be seen (Fig. 1) that, as with the human erythrocyte cholinesterase, there is a marked decline in the rate of hydrolysis of aliphatic substrates as the acyl group is increased in size from acetate to butyrate. The same

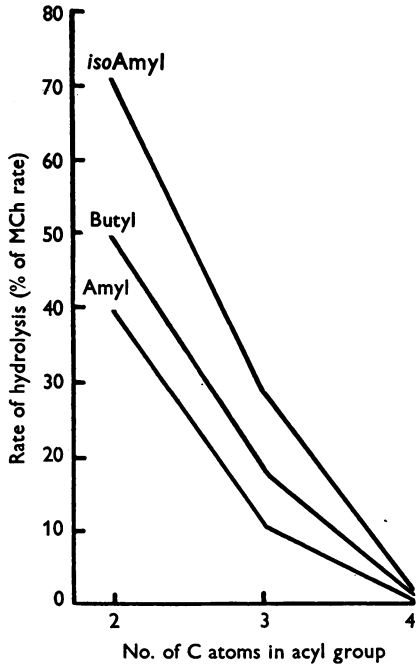
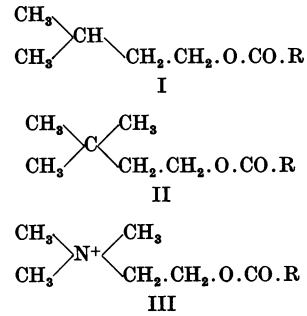


Fig. 1. Effect of changes in acyl group size on the relative rate of hydrolysis of aliphatic esters by horse-erythrocyte cholinesterase.

trend may also be seen with the choline and glyceryl esters (Table 7). In Fig. 2 the characteristic effect of variations in the structure of the alkyl group found with all the cholinesterases so far examined is again seen. Increasing alkyl chain length causes an increase in the rate of hydrolysis up to *n*-butyl; addition of further carbon atoms to the end of the chain (*n*-amyl, *n*-hexyl) leads to a fall in the rate of hydrolysis, but substitution of these carbon atoms in the 3-position as in primary *iso*amyl (I), 3:3-dimethylbutyl (II), in imitation of the choline structure (III), is accompanied by a marked increase in rate of hydrolysis. The effect of changes in alkyl group configuration is closely similar in both the acetate and propionate series but, as expected from Fig. 1, the graph for propionate esters lies below that for acetates.



The values of all but two of the aliphatic esters tested lie within 5% of those for human erythrocytes, the discrepant esters being 3:3-dimethylbutyl

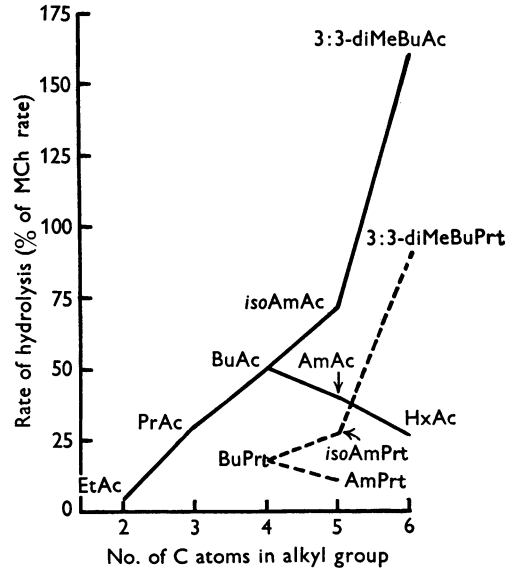


Fig. 2. Effect of alkyl group configuration on the relative rate of hydrolysis of aliphatic esters by horse-erythrocyte cholinesterase. Continuous line, acetates; broken line, propionates.

acetate, 160% (horse), 180% (human) and *n*-amyl propionate, 11% (horse), 6% (human). The species difference between man and horse is therefore much less with the erythrocyte than with the plasma cholinesterase.

Table 7. *Rates of hydrolysis of esters by horse erythrocyte cholinesterase*

(Choline esters, 6 mM, non-choline esters, 100 mM.)

Ester	Rate of hydrolysis (% of MCh rate)
ACh	166*
PrtCh	144
ButCh	2·5
TA	122
TB	0·5
2-EtBuAc	64
BzlAc	74

* ACh and PrtCh show the phenomenon of auto-inhibition. 6 mM is near the optimum substrate concentration ($S_{opt.}$) for PrtCh; the figure given for the rate of hydrolysis is therefore nearly maximal; but $S_{opt.}$ for ACh, if the horse enzyme behaves like the human, is about 1 mM, and the maximum rate of hydrolysis about 300% of the MCh rate.

DISCUSSION

One general conclusion to be drawn from the data presented here is the close similarity of the horse and human erythrocyte cholinesterases. Apart from two discrepancies, the rates of hydrolysis of all those substrates which have been tested on both enzymes agree to within 5%. This similarity extends to the physical behaviour of the enzymes in that they show similar precipitation and adsorption properties. Certain quantitative differences were noticed when the method of purification described above was applied to human erythrocytes, the chief one being the somewhat lower pH (5·8–5·9) required for complete precipitation of the stromata, but these may well be due to differences in the enzymically inert associated material. Horse erythrocyte cholinesterase can therefore be confidently classed as an acetocholinesterase, and the probability that all cholinesterases previously regarded as 'true' or 'e-type' cholinesterases will be found to be acetocholinesterases is increased.

In view of the different specificity patterns of the plasma and erythrocyte cholinesterases there seems little point in trying to decide which of these enzymes is the more specific. As has been pointed out earlier (Adams & Whittaker, 1949), both enzymes are specific in that there is a definite preference for the choline ester-like configuration as judged by rate measurements. On the other hand, it is likely that structures which approach the choline ester configuration less closely sterically than esters of 3:3-dimethylbutyl alcohol, but contain components of choline esters not possessed by aliphatic esters (e.g. positively charged atoms), may be found to have as high or higher affinities for the active centres of the enzyme and possibly also high rates of enzyme-catalysed hydrolysis.

Our conception of specificity will depend, therefore, on how we define the term. If we base it on the total number of esters attacked, we shall regard butyrocholinesterases as less specific than acetocholinesterases; but, as the results with the horse plasma enzyme show, the rates of hydrolysis of these esters may be lower relative to an appropriate standard than with a cholinesterase which has been regarded simply on the basis of the number of substrates attacked as more specific. Enzyme specificity, when not absolute, is not easily susceptible of quantitative definition.

This point is illustrated by the data presented in Table 8, in which the rate of hydrolysis of acetate and butyrate esters by three cholinesterases has been referred to the rate of hydrolysis of the corresponding choline ester. For the human erythrocyte enzyme it has been assumed that the ACh/MCh hydrolysis ratio is 300% under optimum conditions, and the values listed have therefore been obtained by dividing Adams's (1949) results by three. We may assume that the values for the horse erythrocyte are almost the same. It will be seen that many acetates, relative to acetylcholine, are hydrolysed at nearly the same rate by both the enzymes of human blood, although the rates tend to be higher with the erythrocyte enzyme. The acetate:butyrate ratio for the human plasma cholinesterase is about equal for both choline and aliphatic esters, but is lower for glyceryl esters; consequently the rate of hydrolysis of aliphatic esters by the human plasma enzyme is about the same for butyrates or acetates, provided we refer the hydrolysis to the appropriate choline ester, though this ceases to be quite true for glyceryl esters. It will, however, be noticed that the three esters at the bottom of the table are hydrolysed three to six

Table 8. *Hydrolysis of non-choline esters by cholinesterases from different sources*

(Human plasma, data of Adams & Whittaker (1949). Horse plasma, data of Sturge & Whittaker (1950). Human erythrocytes, data of Adams (1949).)

Esters	Relative rate of hydrolysis (expressed as percentage of corresponding choline ester)		
	Human plasma	Horse plasma	Human erythrocytes
3:3-diMeBuAc/ACh	35	—	60
*iso*AmAc/ACh	27	8	24
*iso*AmBut/ButCh	23	6	—
BuAc/ACh	11	4	16
BuBut/ButCh	11	2·5	—
AmAc/ACh	8	3·5	15
AmBut/ButCh	8	3	—
TA/ACh	14	6	42
TB/ButCh	23	10	—
2-EtBuAc/ACh	6	2	22
BzlAc/Ach	4	—	23

times more rapidly (relative to acetylcholine) by the erythrocyte cholinesterase than the plasma. These esters (Fig. 3) have as a common feature of their structure a branch at the carbon atom next to the one involved in the ester link, and this type of branching has been observed by Adams & Whittaker (1949) to reduce rates of hydrolysis by the human plasma enzyme but to raise them (relative to the unbranched structures) with the human erythrocyte.

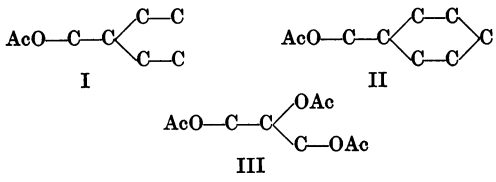


Fig. 3. Carbon skeletons of 2-ethylbutyl acetate (I), benzyl acetate (II), and triacetin (III), to illustrate analogies in structure.

With the horse plasma enzyme the same general tendency is observed as with the human plasma enzyme, but non-choline esters as a class are now much less rapidly hydrolysed than by the erythrocyte enzyme, and this difference is accentuated in the butyrate series because choline esters, but not aliphatic esters, have a lower acetate : butyrate ratio (1 : 2·5 as compared with 1 : 2) than is found with the human plasma enzyme. Non-choline esters, therefore, are hydrolysed in general somewhat less rapidly by the human plasma enzyme, and much less rapidly by the horse plasma enzyme, than they are by the erythrocyte cholinesterases of either species, and from this point of view we should have to regard the horse plasma cholinesterase as the most specific of all of them.

There is now excellent evidence that the characteristic acceleration in rate of hydrolysis through the series *n*-butyl, primary *iso*amyl, 3:3-dimethylbutyl, which has been observed with all cholinesterases so far examined and regarded as characteristic of them, is not due to a fortuitous chemical effect of substituents, but is due to an approach to the choline configuration. First, there is the maintenance of this characteristic pattern in spite of an inversion of the acyl specificity pattern as we pass from aceto- to butyro-cholinesterases. Secondly, the pattern is destroyed and an entirely different one substituted when we pass from a cholinesterase to an ali-esterase derived from the same biological material.

SUMMARY

1. The horse erythrocyte cholinesterase has been partially purified and freed from ali-esterase by a new method.

2. In its specificity and physical behaviour it closely resembles human erythrocyte cholinesterase. It hydrolyses a number of aliphatic esters, the rates of hydrolysis of these substrates being higher the nearer the structure of acetylcholine is approached. The carbon analogue of acetylcholine (3:3-dimethylbutyl acetate) has the highest rate of hydrolysis of any simple aliphatic ester so far examined.

3. The results are discussed in relation to the general question of cholinesterase specificity.

The receipt of a research grant and a research studentship from the Medical Research Council is gratefully acknowledged.

REFERENCES

Adams, D. H. (1949). *Biochim. Biophys. Acta*, **3**, 1.
Adams, D. H. & Whittaker, V. P. (1948). *Biochem. J.* **43**, xiv.
Adams, D. H. & Whittaker, V. P. (1949). *Biochim. Biophys. Acta*, **3**, 358.
Augustinsson, K. B. (1948). *Acta physiol. scand.* **15**, suppl. 52.
Birch, A. J. (1949). *J. chem. Soc.* p. 2721.
Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.
Mendel, B. & Rudney, H. (1943). *Biochem. J.* **37**, 59.
Paléus, S. (1947). *Arch. Biochem.* **12**, 153.
Richter, D. & Croft, P. G. (1942). *Biochem. J.* **36**, 746.
Scheiner, H. (1948). *C.R. Soc. Biol., Paris*, **142**, 36.
Sturge, L. M. & Whittaker, V. P. (1950). *Biochem. J.* **47**, 518.
Whittaker, V. P. (1949). *Biochem. J.* **44**, xlvi.