THE SPECIFICITY OF THE HUMAN ERYTHROCYTE CHOLINESTERASE

by

D. H. ADAMS

Department of Biochemistry, Oxford (England) *

Earlier investigations on cholinesterase specificity were confined to horse serum[16, 17] and human serum[19]. The enzymes from these sources were shown to be non-specific in that they were capable of hydrolysing methyl butyrate and tributyrin, as well as acetyl choline.

Working with human blood, ALLES AND HAWES[4] demonstrated that the substrate specificity pattern of the erythrocyte cholinesterase against certain acetyl esters of choline derivatives differed from that shown by the serum cholinesterase and suggested from this and other evidence that the two enzymes are not identical. RICHTER AND CROFT[14], in an investigation of blood cholinesterases from different species, confirmed the earlier suggestions regarding the non-specific nature of the human serum enzyme. The erythrocyte cholinesterase, on the other hand, was found to be inactive towards tributyrin and methyl butyrate, and these authors concluded that it was specific for acetyl choline. Evidence was obtained however, that the cat, dog, and rabbit erythrocyte enzymes were capable of hydrolysing these aliphatic esters. MENDEL AND RUDNEY[11] divided cholinesterases into two main types which they termed 'true' and 'pseudo' respectively. 'True' cholinesterases, which occur in the brain and erythrocytes of many species, were stated to be active only against acetyl choline and closely related compounds, while the 'pseudo' enzymes, *e.g.*, from horse serum and dog pancreas, are capable of hydrolysing both choline and non-choline esters. It was further stated[10] that 'pseudo' cholinesterases hydrolyse benzoyl choline but not acetyl-β-methyl choline, while the reverse holds for the 'true' enzymes. Cat, dog, and rabbit erythrocytes were included in the 'true' class on the basis of their ability to hydrolyse acetyl-β-methyl choline and failure to attack benzoyl choline, despite the data presented by RICHTER AND CROFT. NACHMANSOHN AND ROTHENBERG[13] suggested that cholinesterases may be divided into 'specific' and 'non specific' types which seem approximately to correspond with MENDEL's 'true' and 'pseudo' classes.

However, apart from the anomaly referred to above, as far as non-choline esters are concerned these conclusions appear to have been based almost exclusively on the failure of certain arbitrarily selected 'true' or 'specific' cholinesterases to hydrolyse tributyrin and methyl butyrate.

BODANSKY[6] claimed that human erythrocyte cholinesterase preparations, freed from the accompanying ali-esterase by the method of MENDEL AND RUDNEY[11] would nevertheless rapidly hydrolyse triacetin. The mouse brain enzyme was also shown to attack this substrate.

* Present address: The London Hospital, London, E.I. (England)

References p. 14.

The specificity data already published on the human erythrocyte cholinesterase is summarized in Table I.

TABLE I

SPECIFICITY OF THE HUMAN ERYTHROCYTE CHOLINESTERASE

Compound	Investigator
A. Compounds hydrolysed	
Acetyl choline	
Acetyl-β-methyl choline	4, 10
Acetyl-α-methyl choline	4
Acetyl erythro-α β-dimethyl choline	4
Acetyl threo-α β-dimethyl choline	4
Glyceryl triacetate	6
Propionyl choline	13
B. Compounds not hydrolysed or hydrolysed only at a very slow rate	
Benzoyl choline	10
Butyryl choline	13
Carbaminyl choline	13
Tributyrin	11, 14
Methyl butyrate	11, 14

Part A of Table I shows that all the compounds so far known to be hydrolysed in the presence of the erythrocyte enzyme are acetates, with the exception of propionyl choline, which is however hydrolysed much less rapidly than the corresponding acetate. No acetates appear in Part B. These data seemed to indicate that the presence or absence of an acetyl group might be of importance in determining whether or not a given substrate is hydrolysed. Consequently a large number of substrates, mainly acetyl esters, have been tested against erythrocyte preparations which have been partially purified to free the cholinesterase from the known accompanying ali-esterase. The majority of the compounds tested are in fact hydrolysed, although in general not at very high rates.

A preliminary report of this work has already been given[3].

EXPERIMENTAL

Source of enzyme

Fresh blood was kindly provided by the E.M.S. blood transfusion service. The cells were separated by centrifuging, washed twice with 0.9% saline and the cholinesterase partially purified by a modification of the method of MENDEL AND RUDNEY[11]. The method described by these workers did not in the author's hands lead to preparations completely freed from ali-esterase activity present in the unpurified material.

The cells from one pint of blood are laked in glass distilled water (500 ml) and shaken with acid washed kieselguhr (65 g). This mixture is filtered through a large Buchner and the solid washed as described by MENDEL AND RUDNEY. After the cake has become almost dry, it is shaken with 300 ml of 0.15% sodium bicarbonate and centrifuged for ten minutes at 2000 r.p.m. The turbid supernatant is removed from the kieselguhr, centrifuged at high speed for two hours, and the resulting clear supernatant discarded.

References p. 14.

The precipitate is suspended in 0.2% bicarbonate, centrifuged once more and finally suspended in 40 ml of 0.2% bicarbonate, giving a turbid, almost colourless, liquid highly active against acetyl choline.

Substrates (see footnote)

Choline esters. Acetyl choline chloride (ACh), butyryl choline chloride (BuCh), acetyl-β-methyl choline chloride (MCh), benzoyl choline bromide (BCh)***.

Non-choline esters

Formates: n-Butyl (BuFo), prim. iso-amyl (isoAmFo).

Acetates: Ethyl (EtAc), n-propyl (PrAc), n-butyl (BuAc), iso-butyl (isoBuAc), n-amyl (AmAc), prim. iso-amyl (isoAmAc), n-hexyl (HxAc)*, benzyl (BzAc), 1:3-dimethyl butyl (1 : 3 DiMeBuAc), 2-ethyl butyl (2EtBuAc), 2-ethyl hexyl (2EtHxAc), mono and triacetin (MA, TA), 3 : 3-dimethyl butyl (3 : 3DiMeBuAc)**.

Propionates: Ethyl (EtPr), n-propyl (PrPr)*, n-butyl (BuPr)*, n-amyl (AmPr), prim. iso-amyl (isoAmPr).

n-Butyrates: Methyl (MeBu), ethyl (EtBu), n-propyl (PrBu), n-butyl (BuBu), n-amyl (AmBu), prim. iso-amyl (isoAmBu), tributyrin (TB).

Inhibitors

1. ββ′-dichlorodiethyl-N-methylamine hydrochloride (DDM).
2. Di-isopropylfluorophosphonate (DFP). (Kindly provided by Dr B. C. SAUNDERS).

Estimation of enzymic activity

Esterase activity was determined manometrically, using AMMON's[5] adaptation of the Warburg technique. (For details see ADAMS AND THOMPSON[1]). In the inhibitor experiments it was found essential to control carefully the time during which the inhibitor and enzyme are incubated in the absence of substrate, in order to obtain reproducible results. This is true of both DDM and DFP (vide MACKWORTH AND WEBB[9] and NACHMANSOHN *et al.*[12] on DFP). As in the previous paper[1] 15 minutes have been allowed. All measurements have been made in μl CO_2/30 minutes (readings taken over the 5–35 minute period).

In the normal procedure followed with the non-choline esters, a sufficient quantity to give a final concentration of approximately 0.1 Molar was pipetted into the side bulbs of the WARBURG vessels, and covered with 0.5 ml of bicarbonate buffer. However, the use of certain esters under these conditions resulted in the occurrence of negative readings during the experiment, particularly on the non-enzymic control manometers, which were not reproducible and gave poor duplicates. The effect was most marked with iso BuAc, EtBu, and EtPr, and was shown to some extent by MeBu, PrPr, PrAc, and BuAc. These esters are all soluble in water to the extent of *ca.* 0.5–2%, and the negative readings were ascribed to a slow solution of ester in the aqueous phase, and contamination

Unmarked = commercial samples.

* Prepared in the laboratory. (n-Hexyl acetate from n-hexyl alcohol and acetic anhydride, n-propyl and n-butyl propionates from the alcohols heated with propionic acid in the presence of 3% sulphuric acid).

** Kindly provided by Dr A J. BIRCH.

*** Kindly provided by the Govt. Experimental Station, Porton, England.

Pure primary iso-amyl compounds could not be obtained, nor any primary-*iso*-amyl alcohol from which to prepare them. Consequently, ordinary 'amyl' esters of commercial origin have been employed as substitutes. These contain *ca.* 90% of primary iso-amyl ester.

All volatile esters were redistilled before use, in most cases after washing with sodium bicarbonate solution. The non-choline esters have been employed throughout at 0.1 Molar concentration and BuCh, MCh, and BCh at 0.03 M.

References p. 14.

of undissolved ester with water, either or both of these processes resulting in a lowering of vapour pressure. Shaking these compounds with the appropriate quantity of bicarbonate buffer at 40° C during 3 hours, followed by a rapid pipetting of the resulting emulsion into the main compartments of the WARBURG vessels (enzyme in the side bulb) served to minimize the effect and reasonably good duplicates were obtained with this modified procedure.

With the more volatile compounds, the N_2 + 5 % CO_2 gas mixture was passed very slowly through the vessels in an effort to prevent undue loss by evaporation.

RESULTS

Evidence for the homogeneity of the enzyme preparations

The question whether the hydrolysis of a number of esters by a tissue preparation is due to a single enzyme can only finally be settled by the isolation and examination of the pure hydrolytic agent. Such procedures usually involve considerable technical difficulties, and, in fact, no pure erythrocyte cholinesterase has ever been prepared. Consequently this problem has been attacked by a combination of two of the classical methods available for the investigation of impure preparations. These are:

1. Summation experiments with mixed esters. It may be shown on classical MICHAELIS theory that if a single enzyme is responsible for the hydrolysis of two different esters, the rate at which a mixture of the two is hydrolysed will lie between the rates of hydrolysis of each separately. On the other hand if two distinct enzymes are present, each hydrolysing one ester, the mixture will be hydrolysed at a rate which is the arithmetical sum of the rates of hydrolysis of each separately. This is of course only true if the two systems are independent, for example, that neither substrate inhibits the other's enzyme.

2. Since no single method can give a completely unequivocal answer inhibitor studies have also been carried out. To strengthen the evidence two different inhibitors have been employed, and the results expressed as the ratio, for each given substrate, of the concentrations of the individual inhibitors required to produce 50% inhibition of enzymic activity (See [1, 2] for details of the method).

Summation experiments

MCh rather than ACh has been employed as a standard in those summation experiments which include a choline ester, for the following reasons.

a. MCh exhibits a classical MICHAELIS activity- substrate concentration curve[10] (and confirmed in this laboratory), and is consequently far less sensitive to small variations in substrate concentration than ACh with its humped (MURRAY-HALDANE) type of curve.

b. It is not clear that a straightforward answer would necessarily be expected to result from the exposure to the enzyme of a mixture of an insoluble or partly soluble non-choline ester and a soluble substrate showing this more complicated type of kinetic behaviour.

c. The lower rate at which MCh is hydrolysed renders it a more convenient substrate.

The results of the summation experiments appear in Table II.

In Group I MCh is taken in conjunction with various acetates. The individual experiments run across the columns. Column (a) expresses the rate of hydrolysis of MCh, column (b) the rate of hydrolysis by the same quantity of enzyme of the acetate ester under investigation, and column (c) the corresponding rate of hydrolysis of a mixture of the two compounds. Although from experiment to experiment the same quantity of enzyme has usually been taken, this practice has not been followed in-

References p. 14.

variably. Column (d) shows the sum [(a) + (b)] of the rates of hydrolysis of the two esters individually, and it will be observed that in no case does the observed rate (c) approach this figure. In fact, in practically every case the observed rate for the mixture lies between the rates for the individual esters, in satisfactory agreement with the supposition that a single enzyme is responsible for the observed hydrolysis. This result is expressed in column (e) which gives the algebraic difference between the 'mixed ester' rate and the rate of hydrolysis of the more rapidly hydrolysed substrate. In one or two cases the mixed esters are hydrolysed slightly more rapidly than either separately, but the effect is hardly significant.

TABLE II

SUMMATION EXPERIMENTS

Group I. Acetyl-β-methyl choline + non-choline acetate esters. (Activities in μl CO_2/30 min)

	(a) MCh	(b) Non choline esters	(c) Mixed esters	(d) (a) + (b)	(e) (a) − (c)
MCh + n-propyl acetate . .	238	64	223	302	—15
	241	71	225	312	—16
	221	64	212	285	— 9
MCh + n-butyl acetate . .	235	119	233	354	— 2
	240	113	237	353	— 3
	232	118	228	352	— 4
MCh + n-amyl acetate . .	244	107	238	351	— 6
	255	118	253	375	— 2
	241	103	226	344	—15
MCh + iso-amyl acetate . .	262	179	263	441	+ 1
	177	132	175	319	— 2
	250	180	247	430	— 3
MCh + benzyl acetate . . .	163	101	159	264	— 4
	245	167	246	412	+ 1
	250	181	254	431	+ 4
MCh + triacetin	189	235	183	426	— 6
	188	235	190	425	+ 2
	150	180	146	330	— 4
MCh + monoacetin	232	136	229	368	— 3
	237	141	221	378	—16
	245	145	230	390	—15

Group II. Acetyl-β-methyl choline + non-choline propionate esters. (Activities in μl CO_2/30 min)

	(a) MCh	(b) Non choline esters	(c) Mixed esters	(d) (a) + (b)	(e) (a) − (c)
MCh + n-amyl propionate .	241	15	152	256	— 89
	242	13	138	255	—104
	232	13	139	255	— 93
MCh + iso-amyl propionate	234	69	210	303	—24
	240	63	212	303	—28
	233	74	204	307	—29

References p. 14.

TABLE II (Continued)

Group III. Ethyl acetate + propionate and butyrate esters. (Activities in μl CO_2/30 min)

	(a) Ethyl acetate	(b) Other esters	(c) Mixed esters	(d) (a) + (b)	(e) (a) or (b) − (c)
EtAc + n-amyl propionate	43	28	37	70	— 6
	41	29	37	71	— 4
EtAc + iso-amyl propionate	27	116	111	143	— 5
	33	123	101	156	—22
	33	127	115	160	—12
EtAc + tributyrin	56	21	56	77	0
	51	22	49	73	— 2
	47	22	46	69	— 1
EtAc + iso-amyl butyrate .	50	19	51	69	+ 1
	47	20	48	67	+ 1

Group II presents the results of summation experiments with MCh + AmPr and isoAmPr, in which, again, no positive summation is observed.

Laked erythrocytes treated according to the original method of MENDEL AND RUDNEY tended to show a small degree of positive summation which appeared most marked in the cases of iso AmAc and BzAc (up to 10%). These preparations were also slightly active against tributyrin, the rate of hydrolysis being 3–4% of the MCh rate. The modified purification procedure already described eliminated the small partial summation but the TB rate was only reduced to 2–2.5% of the MCh rate. An active ali-esterase which readily attacks tributyrin accompanies the cholinesterase is unpurified material, and MENDEL AND RUDNEY[11] claim that its elimination reduced the TB hydrolysis to zero. Evidence will be presented however, that the cholinesterase is capable of hydrolysing TB very slowly, and as a practical test of freedom from ali-esterase no preparations have been used which did not comply with the following conditions:

a. Showing no partial positive summation with MCh and iso AmAc.
b. Having a TB activity less than 2.5% of the MCh activity.

In the third group (Table II) ethyl acetate has been substituted for MCh. It is first shown that no positive summation occurs with AmPr and isoAmPr, which themselves give no positive summation with MCh, and then that there is also no, or very little, positive summation with the low TB and isoAmBu activities. The suitability of EtAc as a subsidiary standard is also borne out by the fact that the activity of unpurified erythrocytes towards this substrate is only *ca.* 15% greater than the activity with purified preparations, indicating that EtAc is only slowly attacked by the ali-esterase occurring in unpurified material.

The results with triacetin appear somewhat anomalous in that although this substrate is hydrolysed more rapidly than MCh the rate of hydrolysis of the MCh + TA mixture is, if anything, slightly below the MCh rate. In all the other summation experiments MCh has been the more rapidly split substrate.

Inhibition experiments

The inhibitory effects of various concentrations of DDM and DFP on the enzymic hydrolysis of ACh, MCh, and the two representative non-choline esters TA and AmAc were determined with a view to providing further evidence for the homogeneity of the enzyme preparations. From the inhibitor results the DDM/DFP I_{50} ratios[1] have been

References p. 14.

calculated using the statistical method developed by ADAMS AND WHITTAKER[2] in connection with the plasma enzyme.

Straight lines result on plotting the reciprocal of the inhibitor concentrations against the reciprocal of the % inhibitions (Figs 1 and 2), a necessary condition for the application of the statistical method. In Figs 1 and 2, the lines drawn through the points are

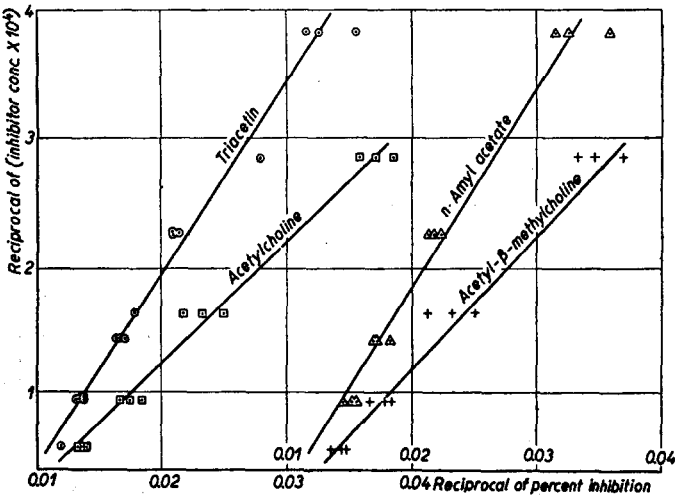


Fig. 1. Inhibition of purified erythrocyte cholinesterase by DDM. The straight lines are the regression lines of ordinates on abscissae. Upper abscissa scale: n-amyl acetate, acetyl-β-methyl choline; lower scale, triacetin, acetyl choline.

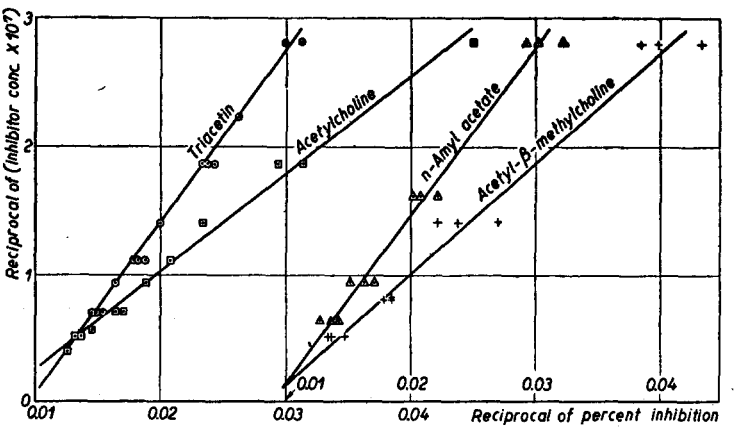


Fig. 2. Inhibition of purified erythrocyte cholinesterase by DFP. The straight lines are the regression lines of ordinates on abscissae; abscissa scales as in Fig. 1.

the lines of closest fit calculated from the data. The most probable values and the upper and lower limits of the I_{50} concentrations for the individual inhibitors are shown in Table III and the corresponding I_{50} ratios in Table IV.

Although there is a greater variation in the I_{50} ratios than might have been expected from the results obtained with the plasma enzyme[2], they formally establish the identity of the enzymes hydrolysing ACh and MCh, and provide further evidence to show that the non-choline esters are also being hydrolysed by one and the same enzyme.

References p. 14.

TABLE III

I_{50} VALUES FOR DDM AND DFP

(Y_{50} values, DDM ($M^{-1} \cdot 10^4$), DFP ($M^{-1} \cdot 10^7$). ($Y_{50} = 1/I_{50}$)

Substrate	Number of observations	Y_{50}	Standard error(s)	Range of Y_{50} for which P = 0.2		I_{50} values and limits outside which less than 20 % of estimates would be expected to fall		
				Y_{50} + ts	Y_{50} — ts	upper	most probable	lower
		DDM						
ACh	12	12.4	0.33	12.9	11.9	8.4	8.05	7.75
MCh	12	12.0	0.45	12.6	11.4	8.75	8.35	7.95
AmAc	12	18.7	0.69	19.7	17.7	5.6	5.35	5.0
TA	16	19.2	0.43	19.8	18.6	5.4	5.2 ($\cdot 10^{-5}$ Molar)	5.05
		DFP						
ACh	12	10.1	0.44	10.7	9.5	10.5	9.9	9.35
MCh	12	9.8	0.45	10.4	9.2	10.9	10.2	9.6
AmAc	12	14.4	0.42	15.0	13.8	7.25	6.95	6.65
TA	14	13.8	0.35	14.3	13.3	7.5	7.25 ($\cdot 10^{-8}$ Molar)	7.0

TABLE IV

I_{50} (DDM/DFP) RATIOS FOR ACh, MCh, AmAc, AND TA

Substrate	Most probable values of I_{50} ratios and limits outside which less than 2 % of estimates would be expected to fall.		
	Upper limit	Most probable value	Lower limit
ACh	900	815	740
MCh	910	820	730
AmAc	845	770	705
TA	770	720	670

The I_{50} ratio variations are considerably smaller than the range of values for the individual inhibitors.

Rate of hydrolysis in relation to substrate configuration

Having demonstrated by summation and inhibition experiments that a single enzyme is responsible for the observed hydrolysis, the preparations were tested against a number of other substrates in an attempt to map out the approximate specificity pattern of the enzyme, and also to establish any possible relationships between substrate molecular configuration and rate of hydrolysis. The collected results are given in Table V.

Several generalization are possible from the data, and these will be considered in detail. The dependence of the rate of hydrolysis on the size of the alkyl group is illustrated in Fig. 3 which shows the rate plotted against the number of C atoms in the n-alkyl chain for a number of acetates, propionates, and butyrates. It will be observed that although the propionates are hydrolysed much less rapidly than the corresponding

References p. 14.

TABLE V

RATES OF HYDROLYSIS OF CHOLINE AND NON-CHOLINE ESTERS IN THE PRESENCE OF PURIFIED ERYTHROCYTE CHOLINESTERASE, EXPRESSED AS PERCENTAGES OF THE RATE OF HYDROLYSIS OF ACETYL-β-METHYL CHOLINE. ALL SUBSTRATES AT 0.1 MOLAR CONCTN. UNLESS OTHERWISE STATED

Substrate	Rate of hydrolysis	Substrate	Rate of hydrolysis
ACETYL-β-METHYL CHOLINE (0.03 M)	100	3 : 3-Dimethyl butyl acetate	180
Acetyl choline (0.006 M)	200	Benzyl acetate	68
Acetyl choline (? M) (estimated peak)	*ca.* 300	Ethyl acetoacetate	6
Benzoyl choline (0.03 M)	1.5	Monoacetin	58
Butyryl choline (0.03 M)	5	Triacetin	125
Ethyl acetate	6.2	Ethyl propionate	2
n-Propyl acetate	29	n-Propyl propionate	9
n-Butyl acetate	49	n-Butyl propionate	16
n-Amyl acetate	45	n-Amyl propionate	5.7
n-Hexyl acetate	22	iso-Amyl propionate	29
iso-Butyl acetate	20	n-Butyl formate	10
iso-Amyl acetate	72	iso-Amyl formate	10
1 : 3 Dimethyl butyl acetate	26	iso-Amyl butyrate	*ca.* 2
2-Ethyl butyl acetate	65	Tributyrin	*ca.* 2
2-Ethyl hexyl acetate	23		

No hydrolysis could be detected with the n-alkyl butyrates from methyl to amyl.

acetates, the maximum in both cases occurs with the n-butyl ester. Fig. 4 illustrates the effect of acyl group size for two groups of fatty acid esters, the n-butyl and iso-amyl series from formate to butyrate. The sharp peak at the acetate point is evident, the propionates being hydrolysed relatively slowly and the butyrates and formates very slowly or not at all.

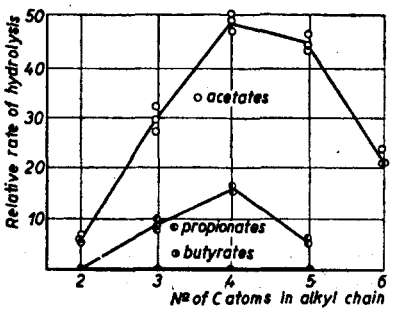


Fig. 3. Effect of alkyl chain length on rate of hydrolysis of n-alkyl esters in presence of erythrocyte cholinesterase. Rate of hydrolysis is expressed as a percentage of rate of hydrolysis of acetyl-β-methyl choline.

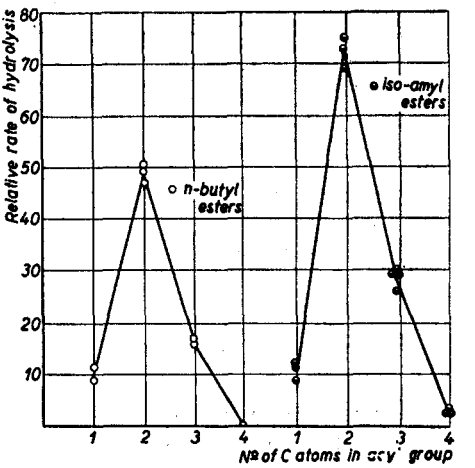


Fig. 4. Effect of acyl chain length on rate of hydrolysis of two series of aliphatic esters in presence of erythrocyte cholinesterase. Rate of hydrolysis is expressed as a percentage of rate of hydrolysis of acetyl-β-methyl choline.

It has already been pointed out that n-butyl acetate is the most rapidly hydrolysed straight chain ester, but Table V shows that it is split at only half the rate of MCh,

References p. 14.

and something like 1/6 of the estimated peak rate of ACh. Extra carbon atoms attached to, or carbon atoms removed from, the end of the alkyl chain lead only to a reduction in the rate. However, extra carbon atoms accommodated as branches may result in a substantial increase. This is illustrated in Table VI which shows the alkyl carbon skeletons of a number of branched chain acetates and the corresponding rates of hydrolysis.

TABLE VI

ERYTHROCYTE CHOLINESTERASE. RELATIONSHIP BETWEEN SUBSTRATE CONFIGURATION AND RATE OF HYDROLYSIS

(Rates of hydrolysis expressed as percentages of the MCh rate)

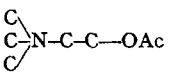
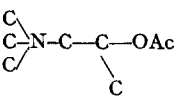
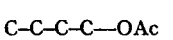
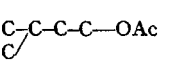
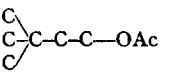
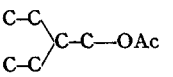
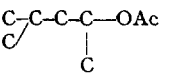
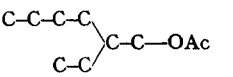
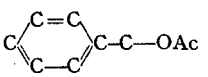
Acetyl choline  *ca.* 300	Acetyl-β-methyl choline  100	
n-Butyl acetate  49	iso-Amyl acetate  72	3 : 3-Dimethyl butyl acetate  180
2-Ethyl butyl acetate  65	1 : 3-Dimethyl butyl acetate  26	2-Ethyl hexyl acetate  23
Benzyl acetate  68		

Table VI indicates that branching at the end of the chain, as in iso-amyl acetate, increases the rate of hydrolysis, and this increase is maintained in 2 EtBuAc where the branching occurs at the 2 position. Benzyl acetate, which may be regarded as being derived from the last compound by ring closure and conjugation is also rapidly attacked. The increase from n-BuAc to isoAmAc, particularly, appeared to indicate the possibility that the more closely the configuration of a given substrate approaches that of acetyl choline the more rapidly it is hydrolysed. In order to test this hypothesis a small quantity of 3 : 3-dimethyl butyl acetate, which may be regarded as the carbon analogue of acetyl choline, was obtained and found to be hydrolysed at approximately 180% of the MCh rate, *i.e.*, more rapidly than any other ester with the exception of ACh itself.

References p. 14.

DISCUSSION

The evidence from summation and inhibition experiments appears to establish beyond reasonable doubt the homogeneity of the enzyme preparations. As has already been pointed out, the I_{50} ratios have not been so closely similar as might have appeared possible from the results of ADAMS AND WHITTAKER[2], but the values for the choline esters are practically identical, and the most probable value of the ratio for the choline esters and for n-amyl acetate are well within each other's ranges. Triacetin falls outside the choline ester values but just inside the AmAc range. Compared with some single inhibitor results which have appeared in the literature as evidence for the homogeneity of enzyme preparations, the agreement between the ratios may be regarded as excellent. Possible reasons for the divergence may be put forward on the basis of SINGER's[15] observations on the inhibitions of a lipase by various non-competitive inhibitors. This author showed that variations in inhibition by a given concentration of a non-competitive inhibitor in the presence of a number of substrates could be correlated with variations in the molecular size of the substrate. DFP certainly inhibits in a manner which is almost entirely non-competitive, and although DDM has on the basis of THOMPSON's[18] earlier work hitherto been classed as competitive there now appears to be considerable doubt regarding this point. The inhibitor appears to resemble DFP in some respects *e.g.*, the degree of inhibition obtained increases considerably with the time of incubation. The variations in inhibition observed using DDM or DFP alone cannot readily be explained in this case on the basis of differences in the molecular size of the substrates, but differences in molecular shape, and the absence of an integral positive charge from the non-choline esters may well act in some manner analogous to that suggested by SINGER. An exact agreement between the I_{50} ratios using arbitrarily chosen inhibitors would therefore be unlikely, and could only be obtained if the relative effects on the individual inhibitors were identical. Nevertheless, it might be expected that the use of the ratio would give a closer agreement between a number of substrates, and that this is true for the system under consideration may be seen by a comparison between the individual inhibitor results in Table III and the ratios in Table IV.

The results confirm the hypothesis that the acetyl group plays a major role in the configuration of rapidly hydrolysed substrates. That the approach of the alcohol group to the choline structure is also of importance seems proven by the very high rate at which the carbon analogue of acetyl choline, 3 : 3-dimethyl butyl acetate, is hydrolysed. This compound is also of interest in that its molecular configuration, shape (including bond lengths) and, consequently, the strength and distribution of the VAN DER WAALS forces holding it in contact with the enzyme, should all be practically identical with the corresponding properties of acetyl choline. It is reasonable to suppose therefore that any differences in the action of the enzyme on the two substrates may be ascribed to the positive charge carried by ACh.

The similar rates at which 2 EtBuAc and isoAmAc are hydrolysed appear to indicate that branching at the 2 position is only slightly less favourable than branching at the end of the chain. Unfortunately however, no 2 : 2-diethyl butyl acetate has been available for comparison with 3 : 3-DiMeBuAc. Branching next to the ester link reduces the rate, as in 1 : 3-DiMeBuAc (Table VI) (derived from iso-amyl acetate by the introduction of a methyl group in the 1 position) which is hydrolysed much less rapidly than the parent compound. ACh and MCh are analogous.

References p. 14.

It has already been mentioned that the enzyme preparations are slightly active against tributyrin, and that the evidence from summation experiments with EtAc given in Table II indicates that the cholinesterase is responsible. In view of the very low activity it has not been possible to carry out any precise inhibitor experiments, but the use of concentrations of DDM and DFP which would be expected to produce a 50% inhibition of the cholinesterase have shown that the degree of inhibition obtained with both inhibitors has not differed very greatly from this figure. The summation experiments with isoAmBu and EtAc also provide evidence for the capacity of the enzyme to hydrolyse butyrates containing a favourable alcohol group, at a slow rate.

Mono and triacetin do not fit readily into the simple hypothesis that closeness of approach to the ACh structure is the sole factor in determining the rate of hydrolysis. TA is the second most rapidly split non-choline ester and suggests that other as yet unknown steric configurations, more or less unrelated to ACh, may be permissible. The somewhat anomalous behaviour of TA in both the summation and inhibition experiments has already been pointed out, and this would seem to indicate that this substrate is associated with the enzyme in some slightly different manner. However its rapid rate of non-enzymic hydrolysis must be borne in mind in attempting an interpretation of the results.

Since the commencement of this work, results by other authors have appeared which are of interest. BOVET NITTI[7] examined the hydrolytic action of snake venom enzyme preparations against acetyl choline and a number of non-choline esters. Her results show that the preparations hydrolysed acetyl choline and all the non-choline acetate esters investigated. Tributyrin and ethyl propionate were not attacked. However no evidence that the choline and non-choline esters were being hydrolysed by the same enzyme was submitted, and although BOVET NITTI appears to assume that they were, she states that the enzyme responsible is not a cholinesterase and refers to it as an 'acetylase'.

HOLTON[8] has confirmed BODANSKY's[6] observation on the hydrolysis of triacetin by brain, and has also provided evidence to show that the snake venom cholinesterase is also capable at least of attacking this substrate.

ZELLER[20, 21] has confirmed and extended the work of BOVET NITTI, showing that the choline and non-choline ester hydrolysis is due to the same enzyme, and proposed that it should be classified as a third ('C') type of cholinesterase in addition to the 'E' and 'S' types already distinguished by ZELLER AND BISSEGER[22].

Despite the wide specificity range of this so-called specific erythrocyte enzyme, the results reported here have demonstrated little or nothing which could be interpreted as evidence that acetyl choline is not, in many respects, a unique substrate. So far as is known at present ACh is hydrolysed at its peak rate nearly twice as rapidly as any other ester, and further, appears to be the only substrate exhibiting a humped (MURRAY-HALDANE) activity-substrate concentration curve, which, since the peak occurs at a relatively low substrate concentration, would particularly adapt this enzyme to the rapid removal of ACh under physiological conditions.

There seems little doubt however that the terms 'true' and 'specific' as originally defined are no longer tenable. The specificity of the brain enzymes has not yet been fully investigated, but there is every reason to believe that the range is much wider than has hitherto been assumed and may well be expected to be as great as that of the erythrocyte enzyme examined here. Neither does there now seem to be any necessity

References p. 14.

for the introduction of ZELLER's 'C' type cholinesterase, originally suggested on the basis of the ability of the snake venom enzyme to hydrolyse non-choline esters. However, certain differences appear when the substrate specificity pattern of this enzyme is compared with that of human erythrocytes. For example, according to BOVET NITTI[7], iso-amyl and n-butyl acetates are hydrolysed only at a very slow rate, and ethyl acetate much more rapidly, a reversal of the results described here.

It is hoped to continue this work by mapping out the specificity pattern of both human plasma and a typical brain enzyme in order to investigate the precise differences in properties which exist between different tissue cholinesterases.

Acknowledgements

I am much indebted to Dr V. P. WHITTAKER for his generous help and advice. I am also grateful to Dr A. J. BIRCH for the gift of 3 : 3-dimethyl butyl acetate prepared by him, and to Dr H. BLASHKO for drawing my attention to the work of BOVET NITTI. Finally I wish to thank the Department of Scientific and Industrial Research, London, for a grant.

SUMMARY

1. The human erythrocyte cholinesterase, hitherto regarded as a highly specific enzyme, has been shown to catalyse the hydrolysis of a large number of non-choline esters.

2. All the acetates investigated have been hydrolysed. The corresponding propionates and formates are attacked less readily, and butyrates very slowly or not at all.

3. In general the more closely the alcohol group simulates the choline configuration, the more rapidly is the ester hydrolysed. Of the substrates investigated, 3 : 3-dimethyl butyl acetate, the carbon analogue of acetyl choline is the most rapidly hydrolysed next to acetyl choline itself.

4. For the differentiation of cholinesterases the terms 'true' and 'pseudo', or 'specific' and 'non-specific', as originally defined, are no longer tenable.

RÉSUMÉ

1. La cholinesterase des érythrocytes de l'Homme, qu'on croyait être une enzyme hautement spécifique, catalyse l'hydrolyse d'un grand nombre d'esters non-choliniques.

2. Tous les acétates examinés sont hydrolysés: les propionates et les formates correspondants sont attaqués moins facilement, et les butyrates très lentement ou pas du tout.

3. En général, plus le groupement alcoolique d'un ester se rapproche de la configuration cholinique, plus l'hydrolyse de l'ester est rapide. L'acétate de 3 : 3-diméthyl butyle, l'analogue carbonique de l'acétylcholine, est le plus rapidement hydrolysé à l'exception de l'acétylcholine elle-même.

4. Les termes 'vrai' ou 'pseudo', 'spécifique' ou 'non-spécifique' qui ont été avancés pour distinguer les différentes espèces de cholinestérases ne peuvent plus être retenus.

ZUSAMMENFASSUNG

1. Es konnte gezeigt werden, dass die Cholinesterase der menschlichen Erythrocyten, die bis jetzt als ein hochspezifisches Enzym betrachtet wurde, in der Lage ist, die Hydrolyse einer grossen Anzahl von nicht-Cholinestern zu katalysieren.

2. Alle untersuchten Acetate konnten hydrolysiert werden. Die entsprechenden Propionate und Formiate werden weniger angegriffen und Butyrate sehr langsam oder garnicht.

3. Im Allgemeinen kann gesagt werden: je mehr die Alkoholgruppe der Cholinform ähnelt, desto schneller erfolgt die Hydrolyse des Esters. Von den untersuchten Substanzen wurde 3 : 3-Dimethylbutylacetat, das Kohlenstoffanaloge des Acetylcholins am schnellsten hydrolysiert, mit Ausnahme des Acetylcholins selbst.

4. Für die Differenzierung von Cholinesterasen sind die Begriffe 'echt' und 'pseudo' oder 'spezifisch' und 'unspezifisch' wie ursprünglich definiert, nicht mehr länger haltbar.

References p. 14.

REFERENCES

1 D. H. ADAMS AND R. H. S. THOMPSON, *Biochem. J.*, 42 (1948) 170.
2 D. H. ADAMS AND V. P. WHITTAKER, *Biochem. J.*, in the press.
3 D. H. ADAMS AND V. .P WHITTAKER, *Biochem. J.*, 43 (1948) 14
4 G. A. ALLES AND R. C. HAWES, *J. Biol. Chem.*, 133 (1940) 375.
5 R. AMMON, *Arch. ges. Physiol.*, 233 (1933) 486.
6 O. BODANSKY, *Ann. N.Y. Acad. Sci.*, 47 (1946) 521.
7 F. BOVET NITTI, *Experientia*, 3 (1947) 283.
8 P. HOLTON, *Biochem. J.*, 43 (1948) 13
9 J. F. MACKWORTH AND E. C. WEBB, *Biochem. J.*, 42 (1948) 91.
10 B. MENDEL, D. B. MUNDEL, AND H. RUDNEY, *Biochem. J.*, 37 (1943) 473.
11 B. MENDEL AND H. RUDNEY, *Biochem. J.*, 37 (1943) 59.
12 D. NACHMANSOHN, M. A. ROTHENBERG, AND E. A. FELD, *J. Biol. Chem.*, 174 (1948) 247.
13 D. NACHMANSOHN AND M. A. ROTHENBERG, *J. Biol. Chem.*, 158 (1945) 653.
14 D. RICHTER AND P. G. CROFT, *Biochem. J.*, 36 (1942) 746.
15 T. P. SINGER, *J. Biol. Chem.*, 174 (1948) 11.
16 E. STEDMAN, E. STEDMAN, AND L. M. EASSON, *Biochem. J.*, 26 (1932) 2056.
17 E. STEDMAN, E. STEDMAN, AND A. C. WHITE, *Biochem. J.*, 27 (1933) 1055.
18 R. H. S. THOMPSON, *J. Physiol.*, 105 (1947) 370.
19 B. VAHLQUIST, *Skand. Arch. Physiol.*, 72 (1935) 133.
20 E. A. ZELLER, *Helv. Physiol. Pharmacol. Acta*, 6 (1948) C 36.
21 E. A. ZELLER, *Advances in Enzymol.*, 8 (1948) 471.
22 E. A. ZELLER AND A. BISSEGER, *Helv. Chim. Acta*, 26 (1943) 1619.

Received August 8th, 1948