THE CHOLINESTERASES OF HUMAN BLOOD

I. THE SPECIFICITY OF THE PLASMA ENZYME AND ITS RELATION TO THE ERYTHROCYTE CHOLINESTERASE*

by

D. H. ADAMS AND V. P. WHITTAKER

Department of Biochemistry, Oxford (England)

INTRODUCTION

It is now well known that the cholinesterase present in human erythrocytes differs in several important respects from that present in human plasma. ALLES AND HAWES[1] were the first to point out that with acetyl choline as substrate, the relation between the initial rate of hydrolysis and the substrate concentration differed for the two enzymes. They also drew attention to certain differences in specificity such as is exhibited, for example, towards acetyl-β-methyl choline. Subsequent workers[2, 3] have regarded the differences in specificity as the most striking, and making much of the fact that the plasma enzyme and cholinesterases from certain other sources hydrolyse tributyrin and methyl butyrate, whereas the erythrocyte enzyme, like the enzymes of nervous tissue, electric organs and muscle, does so only slowly[3] or (when freed from accompanying aliesterase) not at all[2], have referred to the former enzyme as a "non-specific" or "unspecified" esterase[3, 4] or "pseudo" cholinesterase[2], and to the latter as a "specific" or "true" cholinesterase[2, 3].

Recent work from this laboratory[5, 6] has shown that the alleged "specific" character of the human erythrocyte cholinesterase is founded on a misconception of the actual specificity range of this enzyme, and that it is, in fact, capable of hydrolysing a wide range of aliphatic substrates, provided the configuration of the ester molecule is not too widely different from that of acetyl choline itself. It is thus apparent that the specificity range does not in itself constitute a valid basis for distinguishing between these enzymes. The fact that, for example, the plasma enzyme hydrolyses tributyrin whereas the erythrocyte enzyme does not, no longer appears to be due to an inherent inability of the erythrocyte enzyme to hydrolyse non-choline esters as such, but appears to be because the butyryl radical is not an optimum acyl radical for this enzyme. It is possible that the two enzymes have essentially similar specificity patterns, but that butyrates bear the same relation to the plasma enzyme which acetates bear to the erythrocyte enzyme.

This hypothesis seems even more probable if we consider the specificity data for horse serum cholinesterase, an enzyme generally believed to be closely similar to human plasma cholinesterase, but whose specificity has been much more fully studied. EASSON AND STEDMAN[7], using a purified horse serum cholinesterase preparation, showed that

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propionyl choline is hydrolysed about one and three quarters and butyryl choline over twice as rapidly as acetyl choline. Similar results were obtained by GLICK[8], who showed that the rate of hydrolysis falls again with valeryl and caproyl choline, butyryl choline being thus the most rapidly split of the homologous series of choline esters.

These considerations suggested that it might be worth-while to examine in greater detail the specificity of human plasma cholinesterase. Little has been previously done on the specificity range of this enzyme beyond establishing that tributyrin, triacetin, and methyl butyrate[9, 10, 11] are also hydrolysed by it. This work has been reviewed in one of our previous papers[11] in which, however, we showed that there is a small quantity of a second enzyme in unpurified plasma, which appears to contribute to a small, but significant extent to the hydrolysis of triacetin and tributyrin. In the present investigation we have used a preparation of human plasma in which this second esterase has been removed and have investigated its specificity towards some 40 esters. It may be stated at once that the results support the hypothesis that the essential difference between the plasma and erythrocyte enzymes is not in their specificity range but in the size of the acyl group which is optimal in each case. With both, the specificity data in general support the idea that as far as the alcohol part of the ester is concerned, the closer the configuration approaches that of choline, the greater the rate of hydrolysis.

METHODS

Source of Enzyme. Sterile human blood with added anti-coagulant was obtained from the National Blood Transfusion Service. The cells were separated by centrifuging and the plasma freed from a small amount of aliesterase by the method elsewhere described by us[11], or by the method developed for horse serum by STRELITZ[12], up to the point where the cholinesterase fraction is precipitated with ammonium sulphate in stage 2. The enzyme was finally taken up in water and dialysed to remove ammonium sulphate.

Estimation of Enzyme Activity. The rate of hydrolysis of substrates was estimated manometrically[11, 13]. With non-choline esters, the normal technique was to pipette the requisite amount of pure ester into the main compartment of Warburg vessels, then to add enzyme to the side bulb and enough 0.2 % sodium bicarbonate to the main compartment to give a final total volume of 3 ml after tipping. ADAMS' technique[6] was used with a few esters which tended to give anomalous readings with the normal procedure. With choline esters, the ester was usually added to the side bulb and the enzyme to the main compartment. The "effective" concentration of aliphatic substrates (*i.e.*, the concentration that would have been attained if all the ester had dissolved) was 0.1 M, that of choline esters 0.03 M except where otherwise stated. These concentrations were sufficient to saturate the enzyme and the rates of hydrolysis obtained with them are essentially equal to the limiting rates. The enzyme concentration was adjusted to give between 50–200 μl CO_2/30 min. Non-enzymic controls were always included and all data are corrected for non-enzymic hydrolysis.

RESULTS

Evidence for the homogeneity of the plasma cholinesterase preparations. We have previously shown the presence in unpurified plasma of a small quantity of DFP-insensitive aliesterase[11]. This was shown to be present by (a) the small degree of positive summation invariably obtained in experiments with choline and non-choline esters, (b) the incomplete inhibition of aliphatic ester hydrolysis by a concentration of DFP sufficient to produce complete inhibition of acetyl choline hydrolysis, (c) a small hydrolysis of triolein which was shown by summation experiments to compete with the DFP insensitive part of the aliesterase hydrolysis. Purification eliminates this aliesterase activity. Our purified preparations had no triolein activity; concentrations of DFP producing complete inhibition of acetyl choline hydrolysis also produced complete

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inhibition of the hydrolysis of aliphatic esters, and in summation experiments the rate of hydrolysis of a mixture of representative choline and non-choline esters lay between the rates of hydrolysis of the esters measured separately, indicating the presence of a single enzyme[11]. Some typical results are given in Table I. It will be seen that in all but one case, the rate of hydrolysis of the mixed esters lies somewhat below that of the choline ester alone.

TABLE I

SUMMATION EXPERIMENTS WITH ALIPHATIC AND CHOLINE ESTERS

Concentrations of substrates: choline esters 0.03 M, aliphatic esters 0.1 M. For meaning of code letters see Table III.

Carbon dioxide evolution (μl/30 min)			
(a) choline ester	(b) aliphatic ester	(c) mixed ester	c–a
	ACh + TB		
276	126	276	0
272	120	271	− 1
277	132	275	− 2
	BzCh + TA		
317	111	313	− 4
316	105	306	−10
328	109	320	− 8
	BzCh + BuBu		
190	118	182	− 8
192	122	186	− 6
186	115	184	− 2

Purified plasma is found to hydrolyse acetyl-β-methyl choline slowly (2% of acetyl choline rate), if the concentration of enzyme is raised sufficiently to make the rate measurable. That this activity is genuinely due to the action of the plasma enzyme and not to a small amount of erythrocyte cholinesterase is shown by the results of Table II. Here, butyl caproate has been used as the aliphatic substrate; again no positive summation has taken place.

TABLE II

SUMMATION EXPERIMENTS WITH ACETYL-β-METHYL CHOLINE AND AN ALIPHATIC ESTER

Carbon dioxide evolution (μl/30 min)

(a) Acetyl-β-methyl choline (0.03 M)	(b) Butyl caproate (0.1 M)	(c) Mixed esters	c–b
19	75	66	− 9
21	75	66	− 9

The specificity pattern of human plasma cholinesterase. The relation between an enzyme and its substrate is defined by one or more affinity constants and by the rate constant relating the rate of breakdown of substrate to the concentration of enzyme substrate complex. It is the rate constant which is important in specificity studies for

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these may be regarded as attempts to answer the questions, "What compounds are attacked by the enzyme?" and "How rapidly are these compounds attacked?" In general the number of substances capable of combining with the active centres of an enzyme will be larger than those actually breaking down; those combining without breaking down act as inhibitors. While a study of inhibitors may yield valuable information about the configuration of the active centres, we are here concerned with specificity in the narrower sense. Absolute values for the rate constants of substrates cannot be determined without a knowledge of the concentration of the active centres of the enzyme; we have accordingly determined the ratios of the limiting velocities of the various substrates to the limiting velocity of acetyl choline under identical conditions of enzyme concentration, p_H and temperature. These quantities are equal to the ratio of the rate constant of the substrates to the rate constant of acetyl choline. The values obtained for the limiting velocities expressed as a percentage of that of 0.03 M acetyl choline are given in Table III.

TABLE III

SPECIFICITY DATA FOR PURIFIED HUMAN PLASMA CHOLINESTERASE

Figures are limiting rate of hydrolysis expressed as percentage of that of acetyl choline under identical conditions.

Alcohol	Acetates		Propionates		Butyrates	
	Code	Limiting rate of hydrolysis	Code	Limiting rate of hydrolysis	Code	Limiting rate of hydrolysis
Choline	ACh	100			BuCh	210, 206
β-Methyl choline	AcMeCh	1.4			BuMeCh§	12.7, 13.2, 12.6
Triglyceryl	TA	13.5, 14, 14.5			TB	45.5, 44, 47.5
Methyl					MeBu	12, 10.5, 12
Ethyl	EtAc	0.8, 0.9	EtPr	2.6, 2.4	EtBu	6.0, 5.0, 6.0
n-Propyl	PrAc	3.0, 2.7	PrPr*	5.0, 5.0	PrBu	12.0, 11.0, 10.5
n-Butyl	BuAc	11.5, 10.5, 10.0	BuPr*	16.0, 16.5, 15.5, 16.0	BuBu	22.5, 23, 22
n-Amyl	AmAc	8.5, 7.5, 8.0	AmPr	9.5, 9.0, 9.0	AmBu	16, 15.5, 15.5
n-Hexyl	HxAc*	2.3, 1.8			HxBu*	3.0, 3.4
iso-Amyl	isoAmAc	27, 28, 26	isoAmPr	38, 38.5, 36	isoAmBu	46, 45.5, 44, 44
1:3-Dimethyl-n-butyl	1:3-diMeBuAc	2.2, 1.9				
2-Ethylbutyl	2-EtBuAc	5.7				
Benzyl	BzAc	3.3, 4.0				
3:3-Dimebutyl	3:3-diMeBuAc†	35, 35.5	3:3-diMeBuPr†	53		
3:3-MeEtbutyl	3:3-MeEtBuAc†	23, 23	3:3-MeEtBuPr†	24.5, 24.5		

	Valerates		Benzoates		Caproates	
Choline			BzCh	36.5, 36.5, 35.5		
isoAmyl	isoAmVa*	5.6, 5.6			isoAmCa*	9.8, 11.4
n-Butyl	BuVa*	12.6, 11.2			BuCa*	4.9, 4.7
n-Amyl	AmVa*	8, 7.3				

* Prepared in the laboratory † Kindly provided by Dr BIRCH § Kindly provided by Miss NISBET

It will be seen that like the erythrocyte enzyme, the plasma enzyme hydrolyses a wide variety of aliphatic esters at rates which are, in general, less than those of the corresponding choline esters, but which are nevertheless fairly rapid in the case of the more favourable alcohol configurations. In contrast to the erythrocyte enzyme, however, hydrolysis of propionate esters is in each case more rapid than that of the corresponding acetate, and hydrolysis of the corresponding butyrate is more rapid still; this is true

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both for choline and non-choline esters. The effect of increasing chain length in the acyl group for five series of esters is brought out in Fig. 1. It will be seen that in each series an increase in length of the acyl chain leads to a progressive increase in the relative rate of hydrolysis up to butyrate, but that further increase in acyl group size, in those esters which we have had an an opportunity of examining, leads to a sharp fall in activity. These results are in accord with STEDMAN's and with GLICK's results[7,8] for the homologous series of n-acyl cholines and horse serum cholinesterase. The reason for the abnormally slow rate of hydrolysis of isoamyl valerate (which one would expect to be hydrolysed faster than the corresponding caproate) is unexplained.

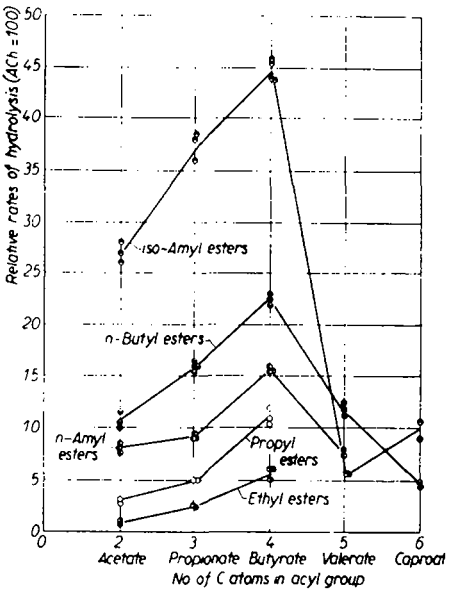

Fig. 1. Effect of acyl group size on rate of hydrolyses of aliphatic esters by plasma cholinesterase

These results are in striking contrast to those obtained with erythrocyte cholinesterase where, it will be recalled, the maximum rate of hydrolysis was obtained, in all the series of esters tested, with acetates, and by which butyrates are hydrolysed only very slowly, if at all. These facts provide an explanation of the well known fact that benzoyl choline is hydrolysed by the plasma enzyme but not by the erythrocyte enzyme. The benzoyl group is even larger than the butyryl group so that we should not expect benzoates to have much chance of being hydrolysed by the erythrocyte enzyme.

Alkyl Specificity. Fig. 2 illustrates the effect of increasing the length of the alkyl chain in three homologous series of n-alkyl esters. As with the erythrocyte enzyme, increase in alkyl chain length up to 4 carbon atoms (*i.e.*, n-butyl esters) results in a progressive increase in the relative rate of hydrolysis; further increases in the length of the carbon chain lead to a falling off in activity.

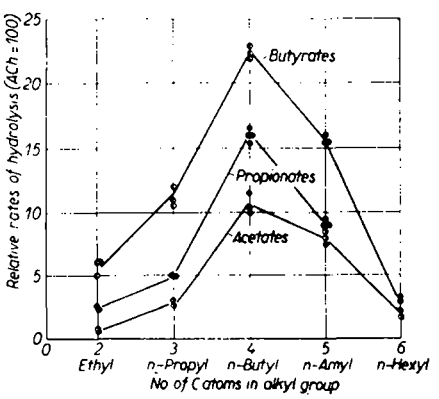

Fig. 2. Effect of chain length of alcohol group on rate of hydrolyses of aliphatic esters by plasma cholinesterase

Effect of Chain branching. Table IV summarizes the effect of chain branching. Although data for propionates and butyrates are not, in every case, available, the results given leave no doubt that whereas addition of further C atoms to the end of the n-butyl chain led (Fig. 2) to a falling off in the rate of hydrolysis, accommodation of these carbon atoms as branches at position 3 (as in isoamyl and 3:3-dimethyl butyl esters) leads to a considerable increase in rate. Once again, as with the erythrocyte enzyme, it is clear that as the choline like configuration is more and more closely approached, the rate of hydrolysis is increased.

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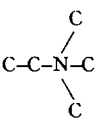
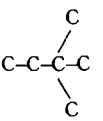
TABLE IV

EFFECT OF CHAIN BRANCHING ON LIMITING RATE OF HYDROLYSIS OF ESTERS BY PLASMA ENZYME

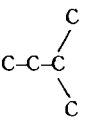
Figures are limiting rates of hydrolysis expressed as percentage of rate of hydrolysis of acetyl choline under identical conditions. (Key to code given in Table III).



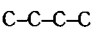
ACh = 100
BuCh = 206



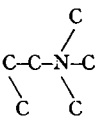
3:3-diMeBuAc = 35
3:3-diMeBuPr = 53



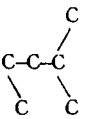
isoAmAc = 27
isoAmPr = 37
isoAmBu = 45



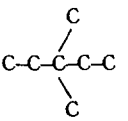
BuAc = 11
BuPr = 16
BuBu = 22.5



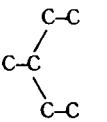
AcMeCh = 1.4
BuMeCh = 12.8



1:3-diMeBuAc = 2



3:3-MeEtBuAc = 23
3:3-MeEtBuPr = 24.5



2-EtBuAc = 5.7

BzAc = 3.5

In one respect the "alcohol" specificity of the plasma enzyme differs from that of the erythrocyte enzyme. Chain branching in the carbon atom next to the ester link (C-1 branching) has a far more adverse effect on the rate of hydrolysis by the plasma enzyme than by the erythrocyte enzyme. Table V shows some comparative results for the two enzymes. The effect of chain branching is shown by the ratio of the rate of hydrolysis of the branched chain compound to that of the parent compound. It will be seen that whereas chain branching at C-1 as in acetyl-β-methyl choline or 1:3-dimethyl butyl acetate depresses the rate of hydrolysis in the presence of the erythrocyte enzyme by only about one-third, these substrates are hydrolysed by the plasma enzyme at less than 2% and 8% respectively of the rate of their parent compounds. The two enzymes also appear to behave differently with respect to chain branching at the 2 position: in the case of the plasma enzyme substitution of an ethyl group in the 2 position in butyl acetate brings about a considerable reduction in the rate of hydrolysis relative to that of butyl acetate itself, whereas this substitution leads to an enhancement of the rate of hydrolysis in the case of the erythrocyte enzyme. Branching at C-3 leads to an increase in the rate of hydrolysis in both enzymes.

It is thus clear that the behaviour of the "specific" substrate, acetyl-β-methyl choline, is not an isolated phenomenon, but is shown by an aliphatic acetate. It was a matter of some interest to see whether the inhibitory effect of C-1 substitution can

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TABLE V

THE EFFECT OF CHAIN BRANCHING ILLUSTRATED BY THE RATIO OF THE RATE OF HYDROLYSIS OF VARIOUS BRANCHED CHAIN ESTERS AND THAT OF THE PARENT ESTER

Esters compared	Ratio of rate of hydrolysis		Esters compared	Ratio of rate of hydrolysis (Plasma enzyme)
	Erythrocyte Enzyme	Plasma Enzyme		
	Branching at C–1			
AcMeCh / ACh	0.33*	0.014	BuMeCh / BuCh	0.062
1:3-diMeBuAc / isoAmAc	0.36	0.075		
	Branching at C–2			
2-EtBuAc / BuAc	1.3	0.52		
	Branching at C–3			
isoAmAc / BuAc	1.5	2.45	isoAmBu / BuBu	2.0
3:3-diMeBuAc / isoAmAc	2.5	1.3		

* Estimated value for ACh at optimum concentration.
Figures for erythrocyte enzyme from ADAMS[6].
Figures for plasma enzyme calculated from averaged data given in Table III.
BuMeCh 0.015 M.
For key to code see Table III.

be reduced by replacing the acetate group by a more favourable acyl group, *e.g.*, butyrate. The last column of Table V shows the rates of hydrolysis by the plasma enzyme of butyryl-β-methyl choline and isoamyl butyrate relative to their parent compounds. It will be seen that whereas branching in the C–3 position leads to the enhancement of the rate of hydrolysis observed with the acetates and interpreted above as due to a closer approach to the choline configuration, C–1 branching leads to a diminution in the rate of hydrolysis which is again of the same order as that observed with the acetates. Thus, although the β-methyl choline ester/choline ester ratio is 4–5 times greater for the butyrates than for the acetates, we must nevertheless conclude that the effect of C–1 substitution is a general one and largely independent of acyl and alkyl group size.

DISCUSSION

Current classifications of the cholinesterases are based on the following assumptions (a) that there are two main types of cholinesterases, those of brain and erythrocytes being prominent examples of the first and those of human and horse serum being prominent examples of the second; (b) that the first type alone have any real claim to be regarded as cholinesterases, the second class hydrolysing choline esters merely as a result of their ability to attack carboxylic esters generally[2, 3, 4].

The results presented in this paper, taken in conjunction with those already published for the erythrocyte enzyme[5, 6] show that while the number of non-choline esters hydrolysed by the plasma enzyme may well be larger than the number hydrolysed by

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the erythrocyte enzyme, the essential difference between them is not primarily one of the specificity range, but lies mainly in the fact that the acyl group which is optimal for the erythrocyte enzyme differs from that which is optimal for the plasma enzyme. Thus, although evidence is forthcoming from other directions against assumption a)[14, 15, 16] our results impugn not it, but assumption b). We agree with ALLES AND HAWES[1] and MENDEL AND RUDNEY[2] that it is unlikely that human plasma cholinesterase contributes significantly to the hydrolysis of acetyl choline in blood and tha tits physiological rôle is obscure, but the specificity data presented above, by demonstrating that there is a marked preference for those aliphatic substrates which approach most closely the choline configuration, show that the plasma cholinesterase has as much right to be regarded as a cholinesterase as the cholinesterase of erythrocytes, though, to be sure, it is not primarily an "acetyl cholinase". Indeed the results with C–1 and C–2 substitution appear to indicate that the permitted deviation from the choline structure is less in the case of the plasma enzyme. Clearly, much more specificity work needs to be done before a rational and comprehensive scheme of classification of the esterases can be drawn up.

A minor, but interesting point emerging from our specificity work is that the rates of hydrolysis of benzoyl choline by the erythrocyte enzyme and of acetyl-β-methyl choline by the plasma enzyme, though very small, are definitely measurable. In each case we have evidence that the hydrolysis is not due to contamination of the enzyme by the cholinesterase of different type. It is unwise, therefore, to attach too much significance to small rates of hydrolysis of these substrates by enzyme preparations or to attempt to use them to measure low concentrations of the enzyme for which they are specific.

The differences between the two cholinesterases of human blood may be summarized as follows:

1. For any given alcoholic group the optimal acyl group for the erythrocyte enzyme is acetate and for the plasma enzyme is butyrate.

2. Both enzymes hydrolyse most rapidly those aliphatic esters which approach most closely the choline configuration, but differ with respect to the effect of chain branching in the carbon atoms of the alcohol adjacent to the ester link. C–1 substitution leads to a fall in the rate of hydrolysis by the erythrocyte enzyme to about one third of the rate of the parent compound in both a choline and an aliphatic ester; in the case of the plasma enzyme the fall is to between 1 and 8% depending on the nature of the parent ester. In the one case of C–2 substitution investigated, an increase in rate was found with the erythrocyte enzyme and a fall with the plasma enzyme.

3. The enzymes show differences in their kinetic behaviour and in the extent to which they are inhibited by certain substances.

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SUMMARY

1. The specificity of human plasma cholinesterase freed from accompanying aliesterase by partial purification has been studied using some 40 choline and non-choline esters.

2. In contrast to the human erythrocyte cholinesterase, the plasma enzyme hydrolyses butyrates most rapidly in any series of esters. The "alcohol specificity" of the enzyme (except in respect of substitution in the carbon atom adjacent to the ester link) is, however, similar to that of the erythrocyte enzyme, aliphatic esters being more rapidly split the more closely they approach the choline configuration.

RÉSUMÉ

1. La spécificité de la cholinestérase du plasma de l'homme, libérée de l'aliestérase qui l'accompagne par une purification partielle, a été étudiée à l'aide d'environ 40 esters choliniques et noncholiniques.

2. Contrairement à la cholinestérase des érythrocytes de l'homme, l'enzyme du plasma hydrolyse les butyrates avec la plus grande rapidité dans toutes les séries d'esters. La "specificité vis-à-vis de l'alcool" de l'enzyme (sauf en ce qui concerne la substitution de l'atome de carbone adjacent à la liaison ester) est cependant proche de celle de l'enzyme globulaire, les esters aliphatiques étant d'autant plus rapidement hydrolysés qu'ils se rapprochent plus de la configuration de la choline.

ZUSAMMENFASSUNG

1. Die Spezifizität der menschlichen Plasma-Cholinesterase wurde nach Abtrennung der begleitenden Aliesterase (durch partielle Reinigung) untersucht und 40 Cholin- und Nichtcholinester verwandt.

2. Im Gegensatz zur Cholinesterase der menschlichen Erythrocyten, hydrolysiert das Plasmaenzym Butyrate am schnellsten in allen Serien von Estern. Die Alkoholspezifizität des Enzyms (ausgenommen hinsichtlich des Ersatzes des Kohlenstoffatoms nahe der Esterbindung) ist jedoch ähnlich dem Enzym der Erythrocyten. Aliphatische Ester werden um so schneller gespalten, je näher sie der Cholinkonfiguration kommen.

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ADDENDUM

PREPARATION OF BUTYRYL-β-METHYLCHOLINE PERCHLORATE

by

J. M. NISBET

β-Methyl choline perchlorate was heated on a water-bath under reflux for three hours with excess redistilled butyryl chloride. The reaction mixture solidified on cooling, and was extracted with dry ether several times and then recrystallized twice from ethanol. Colourless crystals, m.p. 101° C. (Found: C, 41.95; H, 7.70 $C_{10}H_{22}O_6NCl$ requires C, 41.74; H, 7.65 %).

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