THE STRUCTURE OF THE ACTIVE SURFACE OF SERUM CHOLINESTERASE*

by

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In the enzymology of choline esters the surprising fact has been established that besides the so-called "specific" or "true" cholinesterase, which is connected with the conductive process of nerves and muscles[1], a second enzyme, the "pseudo" or "serum" cholinesterase, exists with different characteristics and an as yet undefined physiological role. Although the latter enzyme splits choline esters faster than any neutral substrates, ADAMS AND WHITTAKER[2] concluded from a study of the specificity of both enzymes that "there exists in erythrocyte enzyme a negatively charged atom, which is absent from the active centres of the plasma enzyme".

A most natural explanation for the high affinity of serum cholinesterase for choline esters would certainly consist in the presence of a negative charge within the active surface. We tried to reach a decision about this point by applying to the serum enzyme certain criteria, which were found previously so fruitful in the definition of the various groups inside the active centre of true cholinesterase[3–5]. At the same time we hoped to reach a better understanding of the characteristic differences between the two enzymes.

MATERIALS AND METHODS

The enzyme used throughout these experiments was fraction IV, 6 of human plasma proteins, which was obtained through the courtesy of Dr. RUTH M. FLYNN of the Department of Biological Chemistry of Harvard Medical School. This powder, when diluted with buffer of pH 7.3 in a ratio 1:4000 (w/v), hydrolyzed 6.5 μmoles acetylcholine (ACh)/ml/hour, when the substrate concentration was $5 \cdot 10^{-2}$ M.

Substrates. All alkyl fluoroacetates used in this investigation were synthesized by Dr. J. BLANK of the Research Department of the Israeli Ministry of Defence[6]. Their relevant physical properties have been described by BERGMANN AND SHIMONI[7]. In the present experiments we avoided the use of ethanol to increase the solubility of the neutral substrates and extended the pS-activity curves of the halogenoacetates only to the maximum concentration obtainable in the buffer (see Table I).

The alkyl chloro- and bromo-acetates were commercial samples, which were purified by fractional distillation. For ACh above $5 \cdot 10^{-2}$ M, for the chloro- and bromo-acetates and for the lower fluoroacetates up to the *n*-propyl ester control vessels were run for each concentration, simultaneously with the enzymatic reaction, in order to correct for the appreciable spontaneous hydrolysis. This was unnecessary for the higher esters.

For the *manometric* measurements the buffer used had the following composition: $NaHCO_3$ 0.025 M; NaCl 0.1 M; $MgCl_2$ 0.04 M; gelatine 0.1%. The pH was adjusted to 7.3 before each experiment. The gas phase consisted of air, mixed with 5% CO_2. The vessel with the highest substrate concentration was always checked for pH constancy at the end of the experiment.

* This paper is dedicated to Prof. E. WERTHEIMER, Head of the Department of Biochemistry, Hebrew University, Hadassah Medical School, in honor of his 60th birthday.

References p. 259.

TABLE I

EXPERIMENTAL CONDITIONS FOR THE HYDROLYSIS OF ALKYL HALOGENOACETATES

Compound	*Maximum solubility in bicarbonate buffer (M)*	*Highest substrate concentration used (M)*	*Spontaneous hydrolysis at highest substrate concentration (μmoles/ml-hour)*
a. *Fluoroacetates*			
methyl	$3.3 \cdot 10^{-1}$	$1.1 \cdot 10^{-1}$	
ethyl	$4.2 \cdot 10^{-1}$	$3.6 \cdot 10^{-1}$	3.0
n-propyl	$1.6 \cdot 10^{-1}$	$9.4 \cdot 10^{-2}$	1.3
n-butyl	$3.1 \cdot 10^{-2}$	$2.8 \cdot 10^{-2}$	0.8
n-amyl	$1.3 \cdot 10^{-2}$	$1.2 \cdot 10^{-2}$	0.3
n-hexyl	$3.1 \cdot 10^{-3}$	$2.8 \cdot 10^{-3}$	nil
*iso*propyl	$2.2 \cdot 10^{-2}$	$1.8 \cdot 10^{-2}$	0.4
*iso*butyl	$5.0 \cdot 10^{-2}$	$3.5 \cdot 10^{-2}$	0.3
tert. butyl	$3.1 \cdot 10^{-2}$	$2.8 \cdot 10^{-2}$	nil
b. *Chloroacetates*			
methyl	$3.1 \cdot 10^{-1}$	$1.5 \cdot 10^{-1}$	1.8
ethyl	$9.4 \cdot 10^{-2}$	$8.8 \cdot 10^{-2}$	1.2
c. *Bromoacetates*			
methyl	$2.1 \cdot 10^{-1}$	$2.0 \cdot 10^{-1}$	7.1
ethyl	$7.8 \cdot 10^{-2}$	$5.8 \cdot 10^{-2}$	2.2

For the determination of the pH dependence of substrate hydrolysis or inhibitory effects the *colorimetric* method of HESTRIN[8] was applied. ACh was used in a standard concentration of $1 \cdot 10^{-2}$ *M*, the buffer was 0.1 *M* phosphate for pH < 8.5 and 0.1 *M* borate for pH > 8.5. Samples of 0.3 ml were withdrawn from the reaction mixture after 0, 4, 8, 12 and 16 min and diluted with 0.7 ml water before addition of the hydroxylamine reagent. The rates were plotted on graph paper and extrapolated to zero time.

Inhibitors. The mono-quaternary ammonium salts were commercial samples. Pentamethonium (= pentane-1,5-bis-[trimethylammonium] bromide) and decamethonium were a gift of Allen and Hansbury Ltd., Manchester, England. Both hexa- and nona-methonium were synthesized in our laboratory by Dr. J. KLEIN.

RESULTS

pS-activity curves of halogenoacetates. In Fig. 1–3 we compare the rates of hydrolysis as function of the substrate concentration for ACh and a number of uncharged esters. The most important observation is the bell-shaped curve for ethyl fluoroacetate, since both with true cholinesterase and with liver esterase pS optima were found for most of the halogenoacetates[7]. All other curves appear to be S-shaped similar to ACh, but in view of the solubility limitations accepted for these experiments final judgment must be postponed, until more extended data become available. It can be concluded from the data in Fig. 1 that in the fluoroacetate series affinity increases with increasing length of the alkyl chain. Since the position of the curves does not permit a comparison of rates amongst these esters at the same substrate concentration, we have plotted in Fig. 4 the pS value, which gives an activity of 2 μmoles/ml-hour with an enzyme dilution of 1:2000, as function of the number of C-atoms in the alkyl chain of the fluoroacetates. The pS-curves for the *n*-butyl and *n*-amyl ester in Fig. 1 intersect, and therefore an irregularity is observed in Fig. 4 for $n = 4$ and $n = 5$. For the two lowest members of the series the enzymic rate runs parallel to the rate of spontaneous hydrolysis, but for the higher esters the inverse relationship holds. Evidently, with the high-molecular

References p. 259.

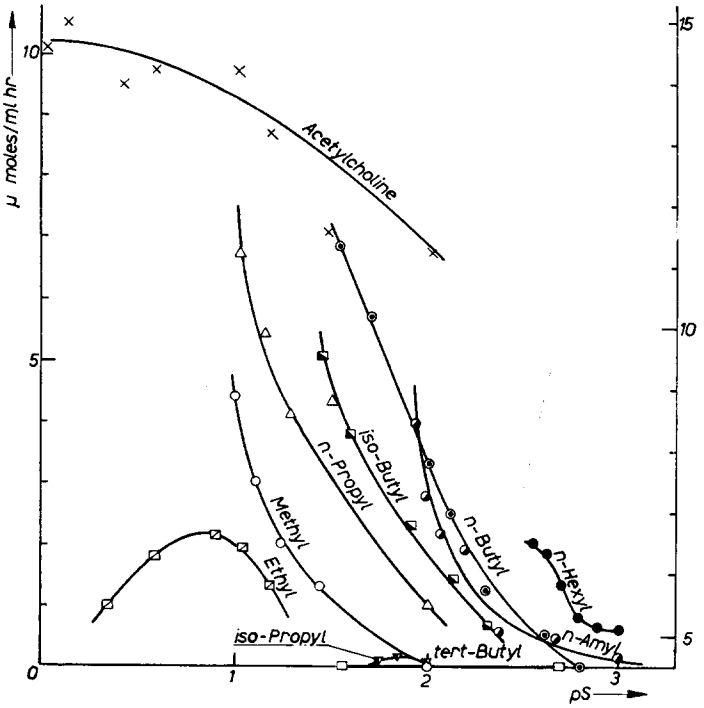

Fig. 1. pS-Activity curves of acetylcholine and alkyl fluoroacetates. Dilution of serum cholinesterase IV–6, used in these experiments:

for ACh	1:4000
for methyl to butyl fluoroacetate	1:2000
for amyl and hexyl fluoroacetate	1:1000
for *iso*propyl and tert. butyl ester	1: 500

For comparison the experimental rates were recalculated for an enzyme dilution 1:2000, since the linear relationship between enzyme concentration and activity was proved for the above range. Right-hand ordinate for ACh only, left-hand ordinate for all other substrates.

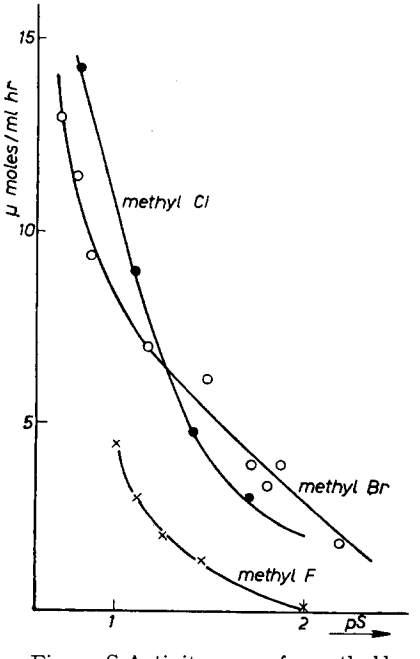

Fig. 2. pS-Activity curves for methyl halogenoacetates. Enzyme dilution 1:2000.

catalyst of the enzymic reaction Van der Waals' forces play an outstanding role. The curve in Fig. 4 is different from the results of STURGE AND WHITTAKER[9], who compared the rates of alkyl acetates and related esters. However these authors did not determine the complete pS-activity curves, but used a constant "effective" substrate concentration for comparison, assuming that the esters dissolved entirely in the reaction mixture.

It is noteworthy that *iso*propyl fluoroacetate shows a small activity, whereas the tert. butyl derivative is practically inactive. This regularity corresponds to our observations with the other esterases mentioned above[7] and establishes the character of the enzymic ester hydrolysis as a S_N2-reaction[10]. On

References p. 259.

the other hand, the tert. butyl derivative inhibits ACh hydrolysis considerably (see Table II, 3). This shows that the tertiary ester combines with the active center, but can not form an "activated" complex to undergo hydrolysis.

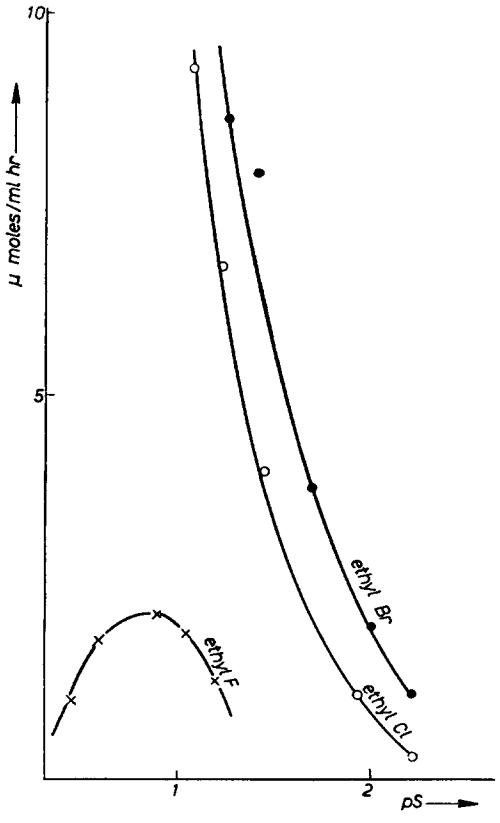

Fig. 3. pS-Activity curves for ethyl halogeno-acetates. Enzyme dilution 1 : 2000.

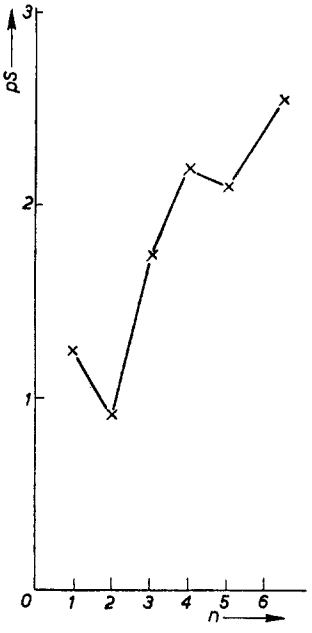

Fig. 4. Affinity of *n*-alkyl fluoroacetates to serum cholinesterase as function of *n*, the number of carbon atoms in the alkyl chain. Ordinate: pS value, at which the enzyme in a dilution of 1 : 2000 hydrolyses 2 μmoles/ml-hour.

Quaternary ammonium salts as inhibitors. This type of inhibitors has been shown previously to be specific for true cholinesterase, but to have no effect on liver esterase[11]. In Table II we report the values for 50% inhibition (= I_{50}) under standardized conditions of two groups of quaternary salts. For the mono-quaternary salts it is at once evident that increasing size of the ion produces increasing inhibition, but in contrast to our results with eel esterase[11] the effect of the tetrabutyl compound is greater than of the tetrapropyl derivative. These data show clearly that the active surface of serum cholinesterase includes an anionic site and thus explain in a simple way the high affinity to choline esters. Single observations, which could have led to the same conclusion, have been reported previously (see *e.g.* KENSLER AND ELSNER[12]), and prostigmine is known since long as one of the most powerful inhibitors of the plasma enzyme[13]. However the significance of these experiments has not been realized.

It is important that inhibition by quaternary ammonium ions is much smaller, when *n*-propyl fluoroacetate serves as substrate (Table II, 1), in contrast to our obser-

References p. 259.

TABLE II

INHIBITORY ACTIVITY OF QUATERNARY AMMONIUM SALTS AND OTHER COMPOUNDS

Serum cholinesterase, 1:2000, and inhibitor were incubated for 45 min before addition of the substrate. Temperature 23°. The figures represent the I_{50} values, unless stated otherwise. The figures for eel esterase are taken from the paper of BERGMANN AND SHIMONI[11].

Compound	*Serum cholinesterase, hydrolyzing*		*Eel esterase, hydrolyzing*	
	ACh $1 \cdot 10^{-2}$ *M*	*n-Propyl fluoroacetate* $1.5 \cdot 10^{-2}$ *M*	*ACh* $4 \cdot 10^{-2}$ *M*	*Diacetine* $4.3 \cdot 10^{-1}$ *M*
1. *Mono-quaternary ammonium salts*				
Tetramethyl	$6 \cdot 10^{-2}$ *M*		$1.5 \cdot 10^{-2}$ *M*	$5 \cdot 10^{-3}$ *M*
Tetraethyl	$5 \cdot 10^{-2}$ *M*	$I_{22} = 1 \cdot 10^{-1}$ *M*	$3 \cdot 10^{-3}$ *M*	$1 \cdot 10^{-3}$ *M*
Tetra-*n*-propyl	$5 \cdot 10^{-3}$ *M*		$1.5 \cdot 10^{-4}$ *M*	$2 \cdot 10^{-4}$ *M*
Tetra-*n*-butyl	$3 \cdot 10^{-3}$ *M*	$3 \cdot 10^{-2}$ *M*	$3 \cdot 10^{-4}$ *M*	$7 \cdot 10^{-4}$ *M*
2. *Bis-quaternary ammonium salts**				
Pentamethonium	$2.9 \cdot 10^{-3}$ *M*		No inhibition up to $1 \cdot 10^{-2}$ *M*	
Hexamethonium	$1.6 \cdot 10^{-3}$ *M*			
Nonamethonium	$1 \cdot 10^{-4}$ *M*		$5 \cdot 10^{-5}$ *M*	
Decamethonium	$4.2 \cdot 10^{-5}$ *M*		$2 \cdot 10^{-5}$ *M*	
3. *Miscellaneous inhibitors*				
Choline	$5.5 \cdot 10^{-2}$ *M*		$4 \cdot 10^{-3}$ *M*	$1.7 \cdot 10^{-3}$ *M*
Prostigmine	$1.6 \cdot 10^{-8}$ *M*		$4 \cdot 10^{-7}$ *M*	
Eserine	$2 \cdot 10^{-8}$ *M*		$6 \cdot 10^{-8}$ *M*	
Glycine	($I_{27} = 1.0$ *M*)		$3 \cdot 10^{-1}$ *M*	
Ethyl glycinate	$3 \cdot 10^{-2}$ *M*		$3 \cdot 10^{-2}$ *M*	
tert. Butyl fluoroacetate	$I_{36} = 2.6 \cdot 10^{-2}$ *M*		$1 \cdot 10^{-2}$ *M*	

* Our results on the inhibition of pseudo cholinesterase by methonium compounds are different from those of PATON AND ZAIMIS[21], who used benzoylcholine as substrate. These discrepancies will be discussed in a subsequent paper.

vations with eel esterase, where the lower members of this series were even more effective inhibitors for the hydrolysis of diacetine than of ACh.

In the series of bis-quaternary ammonium salts appreciable inhibition is already produced by the members with a chain length of 5 or 6 carbon atoms, whereas no effect is found against eel esterase. On the other hand, the increase in the inhibitory effect, when passing from hexa- to deca-methonium, is less pronounced for serum than for eel esterase.

In accordance with the scheme, which we proposed in an earlier paper[3] for the combination true cholinesterase-amino acid esters, the assumption of an anionic site in the pseudo esterase requires that this enzyme too should be unable to hydrolyse such esters, but should be inhibited by them. This is borne out by the data in Table II, 3, where it is also seen that the free acid glycine is a much weaker inhibitor than its ethyl ester. In this connection it should be recalled that ethyl glycinate is a good substrate of liver esterase[14].

pH dependence of substrate and inhibitor activities. The pH-activity curve for the system ACh-serum cholinesterase has been measured previously by various authors[15,16]. We have repeated this measurement by application of the colorimetric method (Fig. 5) and observed a broad optimum between pH 7.5 and 8.4. The problem of the peculiar shape of this curve, which is also characteristic for true cholinesterase, will be dealt with in a separate paper.

References p. 259.

Inhibitors, which form an electrostatic bond with the negative site of the active center, but have no chemical affinity to the esteratic site, reflect in their pH dependence essentially the state of dissociation of the anionic site[4]. If, in addition, polar groups

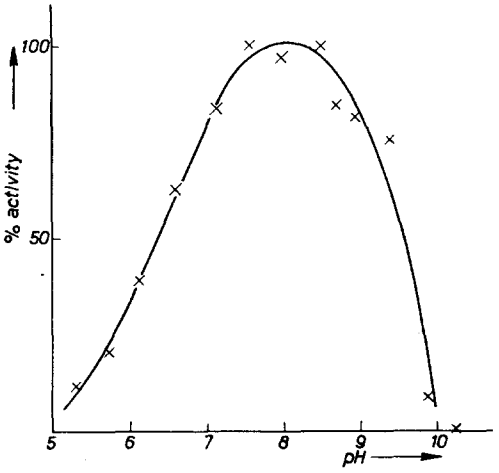

Fig. 5. pH-Activity curve of ACh. Enzyme dilution 1:700, ACh $1 \cdot 10^{-2}$ *M*. Bath temp. 36.5°.

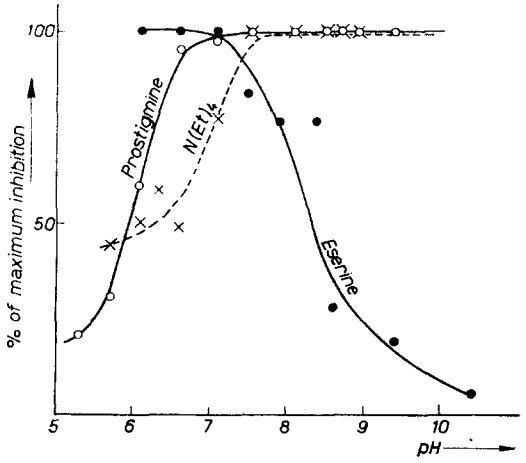

Fig. 6. pH Dependence of the inhibitory effect of eserine, prostigmine and tetraethylammonium ion. Eserine: $5 \cdot 10^{-8}$ *M*, enzyme 1:1000, temperature 23°. Prostigmine: $3.5 \cdot 10^{-8}$ *M*, enzyme 1:700, temp. 36.5°. Tetraethylammonium: $7.5 \cdot 10^{-2}$ *M*, enzyme 1:700, temp. 36.5°. Enzyme and inhibitor were incubated for 30 min before addition of the substrate, $1 \cdot 10^{-2}$ *M* ACh.

are present in the inhibitor molecule, which produce some kind of interaction with the esteratic site, the changes of the latter, which are provoked by changes in pH, will also contribute to the overall variation of the inhibitory effect as function of pH. As shown in Fig. 6, the effect of both prostigmine and tetraethylammonium ion, when measured under non-competitive conditions (*i.e.* at zero time), approaches zero, when the pH *decreases* towards 5. However, the slope of the two curves is different, the prostigmine curve descending at lower pH values. On the other hand, the tertiary base eserine ($pK = 8.1$)[5] shows a decline of activity, when the pH *increases* from 6 to 9, in accordance with the fact that the free base is a much weaker inhibitor than the ionized form.

DISCUSSION

Our experiments demonstrate that the active surface of serum cholinesterase, like that of true cholinesterase, is composed of an esteratic site and, in its immediate neighbourhood, a negatively charged group, the "anionic site", which ionizes completely when the pH increases from 5 to 7. The evidence rests on the following facts:

1. Quaternary ammonium salts inhibit the hydrolysis of both cationic and neutral esters. This effect is proportional to the size of the quaternary ion.

2. Glycine ester, in which the positively charged ammonium group is linked to the ester carbonyl without the mediation of an ether bridge, combines with the active center and serves as inhibitor, but cannot be hydrolyzed.

References p. 259.

3. The inhibitory effect of tetraethylammonium ion, when measured under non-competitive conditions, decreases with *decreasing* pH.

4. The inhibitory effect of the tertiary base eserine decreases with *increasing* pH.

Since serum cholinesterase has thus been shown to contain both an anionic and an esteratic site, it follows that the combination of these groups in the active surface is not sufficient to produce a bell-shaped pS-activity curve with a cationic substrate, as has been claimed by ZELLER AND BISSEGGER[17]. Thus the special feature of true cholinesterase, which presumably possesses two anionic sites[18], is again being stressed. On the other hand, the neutral substrate ethyl fluoroacetate shows a pS optimum with both cholinesterases.

It is of interest to consider the reasons which led ADAMS AND WHITTAKER[2] to reject the anionic site as an important feature of the active center of serum cholinesterase. They found the ratio of affinities of choline for true and pseudo enzyme as 29, using $1.2 \cdot 10^{-2}$ *M* ACh as substrate. If we compare first the I_{50} values for choline and tetraethylammonium (Table II), we obtain a ratio of 1.3 for true and of 1.1 for pseudo cholinesterase, which are practically identical. Furthermore, we find the Michaelis-Menten constant for the system ACh-serum cholinesterase as $6 \cdot 10^{-3}$ (neglecting for the time being the acid and base dissociation constants of the esteratic site), which is about twenty times greater than the corresponding value for eel esterase, $3 \cdot 10^{-4}$ [19]. The decreased affinity of choline towards serum cholinesterase is thus directly related to the decreased affinity of ACh.

There are practically no spatial restrictions for the combination of serum cholinesterase with substrates and inhibitors. This is well demonstrated by the considerable decrease in the inhibitory effect of quaternary ammonium salts, when neutral esters serve as substrates (Table II, 1). Apparently, when electrostatic attraction is missing and the substrate not forced into a definite direction, the substrate molecule can attach itself to the active center in various positions and thus avoid interference with the inhibitor to a certain degree. Similar observations have been made by MCNAUGHTON AND ZELLER for the inhibition of chloroacetate hydrolysis by eserine[20].

The lack of spatial limitations on the surface of serum cholinesterase is also reflected by the behaviour of the methonium compounds: The differences in this homologous series are based entirely on the increasing Van der Waals' forces. Therefore a simple relationship exists between chain length and I_{50} values. For eel esterase, on the other hand, the lower members of the series are practically ineffective and an enormous increase in affinity is observed when the "optimal" chain length is approached. If we assume that the negative charge in both esterases is represented by the same chemical group (*e.g.* a COO^--group), then the Coulombic force between the anionic site and a given quaternary ammonium ion is always the same, if the minimum distance between enzyme and inhibiting ion is equal. Therefore differences in affinity towards either cholinesterase must be due to environmental factors (*e.g.* different degrees of folding of the peptide chain).

The differences in the inhibitory activity of methonium compounds towards the two cholinesterases may lead to an explanation of the curious "localization" of their pharmacological action: Penta- and hexa-methonium serve as ganglionic blocking agents, whereas with decamethonium neuro-muscular block is the most pronounced effect, although ganglionic block is an accessory phenomenon[21]. The presence of pseudo cholinesterase in conductive tissues (*e.g.* in sympathetic ganglia) has been demonstrated

References p. 259.

by ORD AND THOMPSON[22] and by KOELLE[23, 24]. In various organs a mixture of the two cholinesterases may be present, but it is also conceivable that a whole array of enzymes exists with characteristics intermediate between the two extreme types of pure cholinesterases.

The inhibitory effect of tetra-alkylammonium salts increases with increasing molecular size. These observations parallel in a general way our results with true cholinesterase[11] and indicate that our conclusions on the possible role of the latter enzyme in the conductive process apply also to serum cholinesterase, although this enzyme has a much lower turnover number and thus can probably participate only in conduction processes of relatively slow speed. If we assume—as appears probable for thermodynamic reasons—that the serum enzyme can catalyze the reaction

$$ACh + H_2O \rightleftarrows \text{choline} + \text{acetate}$$

in both directions, the system pseudo cholinesterase-ACh (or whatever the "natural" substrate may be) can function as enzymic buffer to regulate the pH of the proteins involved in the conductive process and thus their ion-exchange properties.

SUMMARY

1. Purified human serum cholinesterase combines in its active surface an anionic and an esteratic site, similar to true cholinesterase. The differences between the two types of cholinesterases can be ascribed to a) the presence of at least two negative sites in the true enzyme, b) the lack of spatial restrictions around the active center of the serum enzyme.

2. Methonium compounds show quantitative differences in their inhibitory power towards the two enzymes. The lower members, C_5 and C_6, are active only against the serum enzyme, the higher ones, *e.g.* C_{10}, also against true cholinesterase. These differences suggest an explanation of the curious "localization" of the pharmacological effects of lower and higher methonium compounds.

3. The results indicate that serum cholinesterase behaves principally similar to the true enzyme and thus can play a similar role in nerve conduction, although it participates probably only in relatively slow conductive processes.

RÉSUMÉ

1. La cholinestérase du sérum humain purifiée, de même que la cholinestérase vraie, présente à sa surface active une région anionique et une région estérasique. Les différences entre les deux types de cholinestérases viennent a) de la présence dans l'enzyme vrai d'au moins deux régions négatives, b) du manque de restrictions spatiales autour du centre actif dans l'enzyme du sérum.

2. Les composés méthonium n'ont pas le même pouvoir inhibiteur vis à vis des deux enzymes. Les membres inférieurs de la série, en C_5 ou C_6, ne sont actifs que sur l'enzyme du sérum, les membres supérieurs, par exemple en C_{10}, inhibent également la cholinestérase vraie. Ces différences permettent peut-être d'expliquer la "localisation" curieuse des effets pharmacologiques des composés méthoniums inférieurs et supérieurs.

3. La cholinestérase du sérum se comporte en gros comme l'enzyme vrai et peut donc jouer un rôle semblable dans la conduction nerveuse, quoiqu'elle ne prenne probablement part qu'à des phénomènes de conduction relativement lents.

ZUSAMMENFASSUNG

1. Gereinigte Serumcholinesterase des Menschen vereinigt wie echte Cholinesterase in seiner aktiven Oberfläche eine Anionen- und Esterasenstelle. Die Unterschiede zwischen diesen beiden Cholinesterasenarten können a) der Anwesenheit von mindestens 2 negativen Stellen in dem echten Enzym und b) dem Fehlen räumlicher Beschränkung um das aktive Zentrum des Serumenzyms zugeschrieben werden.

2. Methoniumverbindungen zeigen in ihrer Hemmwirkung gegenüber den beiden Enzymen quantitative Unterschiede. Die niedrigeren Glieder C_5 und C_6 sind nur gegenüber dem Serumenzym wirksam, die höheren z.B. C_{10} ebenfalls gegenüber echter Cholinesterase. Diese Unterschiede legen

References p. 259.

eine Erklärung der seltsamen "Lokalisierung" der pharmakologischen Effekte der niedrigeren und höheren Methoniumverbindungen nahe.

3. Die Ergebnisse zeigen, dass Serumcholinesterase sich hauptsächlich ähnlich wie das echte Enzym verhält und daher bei der Nervenleitung eine ähnliche Rolle spielen kann, obwohl es wahrscheinlich nur an relativ langsamen Leitvorgängen teilnimmt.

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