activity. Only higher concentrations of glutathione appreciably activated aconitase; there was no simple Michaelis–Menten relationship between the concentration of glutathione and the enzyme activity.

6. The conclusion was drawn that the active form of aconitase is either an enzyme–Fe^{2+}–reducing agent or an enzyme–Fe^{2+}–activator complex.

It is a pleasure to express my thanks to Sir Rudolph Peters, F.R.S., Dr R. B. Fisher and Dr R. Cecil for their interest and advice during this work. Thanks are also due to Miss S. Read for skilled technical assistance.

REFERENCES

Buffa, P. & Peters, R. A. (1949). *J. Physiol.* **110**, 488.
Dickman, S. R. & Cloutier, A. A. (1951). *J. biol. Chem.* **188**, 379.
Krebs, H. A. & Eggleston, L. V. (1944). *Biochem. J.* **38**, 426.
Kubowitz, F. (1935). *Biochem. Z.* **282**, 277.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Malachowski, R. & Maslowski, M. (1928). *Ber. dtsch. chem. Ges.* **61**, 2521.
Mathews, A. P. & Walker, S. (1909). *J. biol. Chem.* **6**, 299.
Mohamed, M. S. & Greenberg, D. M. (1945). *Arch. Biochem.* **8**, 349.
Morrison, J. F. (1954*a*). *Biochem. J.* **56**, 99.
Morrison, J. F. (1954*b*). *Biochem. J.* **56**, xxxvi.
Pucher, G. W., Sherman, C. C. & Vickery, H. B. (1936). *J. biol. Chem.* **113**, 235.
Purr, A. & Weil, L. (1934). *Biochem. J.* **28**, 740.
Schubert, M. (1932). *J. Amer. chem. Soc.* **54**, 4077.
Smith, E. L. (1951). *Advanc. Enzymol.* **12**, 191.

The Relationship of Quaternary Ammonium Salts to the Anionic Sites of True and Pseudo Cholinesterase

By F. BERGMANN and RUTH SEGAL
Department of Pharmacology, The Hebrew University—Hadassah Medical School, Jerusalem, Israel

(*Received* 10 *May* 1954)

In recent experiments on serum cholinesterase (Bergmann & Wurzel, 1954) evidence for the presence of an anionic site near the esteratic site of the enzyme was obtained and the conclusion was reached that the active surface of this enzyme is essentially similar to that of true cholinesterase. Since the differences between these two esterases have in the past been ascribed to the absence or unimportance of a negative site in the pseudo enzyme (Adams & Whittaker, 1950), it now becomes necessary to explain on a new basis the specific substrate affinity to either enzyme.

Paton & Zaimis (1949), in their study on alkane bistrimethylammonium salts ('methonium' compounds), reported only little inhibitory activity towards serum cholinesterase, but pronounced activity against laked erythrocytes of rabbits. The results of Bergmann & Wurzel (1954), obtained under different experimental conditions, were not in accord with their findings and indicated that the ratios of affinities of inhibitors of the methonium series towards the two enzymes reverse with increasing chain length. It was expected that a quantitative study of this problem would not only clarify the differences reported, but also contribute to a more precise definition of the active surface of the two cholinesterases and to an understanding of their physiological role. We have therefore investigated the inhibitory effect of two homologous series of ammonium derivatives, viz. monoacidic and diacidic quaternary ammonium bases with the nitrogen attached to the ends of unbranched paraffin chains, in order to evaluate quantitatively the factors determining the intermolecular forces between these enzymes and their inhibitors.

MATERIALS AND METHODS

Substrates. Commercial acetylcholine bromide (ACh) was used. Benzoylcholine chloride (BCh) was obtained through the courtesy of Dr Aeschlimann, Hoffmann-La Roche, Nutley, New Jersey. Diacetin was supplied by British Industrial Solvents Ltd., London.

Inhibitors. Butane-1:4-bistrimethylammonium dibromide ('tetramethonium') and heptamethonium were a gift of Dr H. J. Barber of May and Baker Ltd., Dagenham, Essex, and decamethonium of Messrs Allen and Hanburys Ltd., Manchester, England. All other methonium compounds were synthesized in our laboratory by a method to be published elsewhere. Monoacidic quaternary ammonium salts were prepared from trimethylamine and alkyl halides in dilute ethanolic solutions. The lower members, methyl to *n*-pentyl, were crystallized as bromides. However, the halides of higher members of the series proved to be too hygroscopic for purification. It was, therefore, necessary to use the perchlorates, the properties of which are summarized in Table 1.

Enzyme preparations. Purified, crystalline cholinesterase of human plasma (fraction IV-6-3) was obtained through the courtesy of Dr Ruth M. Flynn, Department of Biochemistry, Harvard Medical School (a brief description of the preparation of this material is given by Edsall, 1947). This material, in a dilution of 1:10000 and at 37°, hydrolyses 9 μmoles/ml./hr., when ACh, 6×10^{-2}M, is used as substrate. Cholinesterase from the electric organ of *Electrophorus electricus* ('eel esterase') was purified according to Rothenberg & Nachmansohn (1947). The solution so obtained, in a dilution of 1:1000, hydrolyses at 23° 5 μmoles/ml./hr., when ACh, 4×10^{-3}M, serves as substrate. Activities were measured under the conditions given in the following paragraph.

The enzyme solution was incubated with the inhibitor for 45 min., before the substrate was added, and the hydrolytic activity was measured manometrically. The buffer used as medium for these experiments contained 0·1M-NaCl, 0·026M-$NaHCO_3$ and 0·04M-$MgCl_2$. The pH was adjusted to 7·3. The gas phase consisted of 95% air and 5% CO_2. The I_{50} values, i.e. the concentrations of inhibitors producing 50% inhibition, were obtained graphically by plotting on semilogarithmic paper the percentage inhibition as a function of inhibitor concentration.

RESULTS

In Table 2 the inhibitory activity of compounds ranging from tetra- to dodeca-methonium is compared. The values for penta- and hexa-methonium against eel esterase were determined with crystalline salts, whereas earlier experiments (Bergmann & Wurzel, 1954), had been made with commercial preparations (Allen and Hanburys Ltd.). The I_{50} value of any reversible, competitive inhibitor depends on the substrate concentration according to equation 1, namely

$$\frac{v_0}{v_i} = 1 + \frac{I \times K_m}{K_i(K_m + S)}, \qquad (1)$$

Table 1. *Properties and analyses of alkyltrimethylammonium perchlorates*

Analyses were performed according to the method of Bodenheimer & Weiler, details of which will be described elsewhere. However, the conditions are as follows: 0·5–1·0 mg. of the organic perchlorate is dissolved in water and added to a solution of copper–pyridine–nitrate complex of known concentration. After the copper–pyridine–perchlorate has precipitated completely (during 12 hr. standing), the supernatant is removed and its extinction compared with that of the original reagent solution. The difference permits the evaluation of the perchlorate concentration in the sample by comparison with a calibration curve, obtained with analytically pure potassium perchlorate.

Alkyl group	M.p. (°)	Solvent for recrystallization	Crystal form	% ClO_4^- Calc.	% ClO_4^- Found
Hexyl	153	*n*-Propanol	Prisms	40·7	40·5
Heptyl	126	Ethyl acetate	Prismatic columns	38·5	38·6
Octyl	118	Ethanol	Plates	36·5	36·5

Table 2. I_{50} *values for monoacidic and diacidic quaternary ammonium compounds*

All salts are derived from trimethylamine and vary only in the length of the carbon chain of the fourth substituent, which is given in the first column by *n*, the number of carbon atoms in the chain. All experiments were carried out at 37°, apart from the system true cholinesterase–monoacidic bases, which was examined at 23°.

	Serum cholinesterase			
	Acetylcholine		Benzoylcholine,	True cholinesterase, acetylcholine,
n	6×10^{-2}M I_{50} (M)	4×10^{-3}M I_{50} (M)	4×10^{-3}M I_{50} (M)	4×10^{-3}M I_{50} (M)
Diacidic bases (methonium compounds)				
4	$4·7 \times 10^{-2}$	$1·8 \times 10^{-3}$	—	$8·6 \times 10^{-2}$
5	$2·1 \times 10^{-2}$	$2·5 \times 10^{-3}$	—	$4·8 \times 10^{-2}$
6	$1·1 \times 10^{-2}$	$9·7 \times 10^{-4}$	$7·3 \times 10^{-3}$	$1·9 \times 10^{-2}$
7	$3·4 \times 10^{-3}$	$4·0 \times 10^{-4}$	—	$1·2 \times 10^{-2}$
8	$1·9 \times 10^{-3}$	$3·0 \times 10^{-4}$	$1·7 \times 10^{-3}$	$2·4 \times 10^{-3}$
9	$6·2 \times 10^{-4}$	$1·0 \times 10^{-4}$	$1·0 \times 10^{-3}$	$3·0 \times 10^{-4}$
10	$3·5 \times 10^{-4}$	$4·6 \times 10^{-5}$	$7·0 \times 10^{-4}$	$2·0 \times 10^{-5}$
12	$1·2 \times 10^{-4}$	—	—	$8·2 \times 10^{-6}$
Monoacidic bases				
1	—	—	—	$1·5 \times 10^{-2}$
2	$1·9 \times 10^{-1}$	—	—	$6·8 \times 10^{-3}$
3	$1·4 \times 10^{-1}$	—	—	$3·0 \times 10^{-3}$
4	$5·4 \times 10^{-2}$	—	—	$2·0 \times 10^{-3}$
5	$1·7 \times 10^{-2}$	—	—	$1·5 \times 10^{-3}$
6	$1·6 \times 10^{-2}$	—	—	$6·9 \times 10^{-4}$
7	$4·3 \times 10^{-3}$	—	—	$6·0 \times 10^{-4}$
8	$2·5 \times 10^{-3}$	—	—	—

where v_0 and v_i denote the rate of enzymic hydrolysis in the absence and presence of inhibitor respectively, S and I the molar concentration of substrate and inhibitor, K_m the Michaelis–Menten constant and K_i the equilibrium constant for the enzyme–inhibitor complex. Therefore figures for two different values of S are included in Table 2.

When pI_{50} ($-\log_{10} I_{50}$), was plotted as a function of n (= the number of carbon atoms separating the charged end-groups), a straight line was obtained for serum cholinesterase, whereas an S-shaped curve characterizes the eel esterase (Fig. 1). A simple law must therefore apply to the system serum-cholinesterase–methonium compounds, which can be formulated as follows: if in equation 1 we set $I = I_{50}$, i.e. $v_0 = 2v_i$, we obtain the relationship

$$K_i = I_{50} \frac{K_m}{K_m + S}, \qquad (2)$$

and thus for a given constant substrate concentration

$$\frac{{}^{n}I_{50}}{{}^{n-1}I_{50}} = \frac{{}^{n}K_i}{{}^{n-1}K_i}. \qquad (3)$$

Therefore $\log K_i$, like $\log I_{50}$, should be a linear function of n, i.e. the difference between successive $\log K_i$ values is constant. We may now derive directly the following equation:

$$RT\,(\log {}^{n}K_i - \log {}^{n-1}K_i) = RT(\log {}^{n}I_{50} - \log {}^{n-1}I_{50}) = \Delta F_{n-1} - \Delta F_n = \text{const.} \qquad (4)$$

We are thus justified in calculating directly from the slope of the straight lines in Fig. 1 the increment in free energy change, $\Delta\Delta F$, accompanying the addition of one —CH_2— group to the central chain of a methonium compound. This magnitude measures the energy gained by transfer of one aliphatic carbon atom from its free state in solution to the state of adsorption on the protein surface. At 310° K we obtain a value of about 500 cal./—CH_2— group. The identical slope, obtained for serum cholinesterase at two different substrate concentrations, shows that $\Delta\Delta F$, as expected, is independent of S.

These observations demonstrate a simple relationship for the system serum-cholinesterase–methonium compound. All other forces between inhibitor and enzyme being kept constant within this homologous series, each additional carbon atom produces the same increase in affinity. Thus each —CH_2— group is held by identical forces. Therefore, the structure of the active surface of this enzyme must be relatively 'simple', i.e. no steric restrictions are imposed on unbranched paraffin chains, at least up to 10 carbon atoms.

The sigmoid curve, shown in Fig. 1 for the system true cholinesterase–methonium compounds, must be due to some factor which 'disturbs' the simple relationship observed with the plasma enzyme in such a way that the affinity of an additional carbon atom in the paraffin chain is at first smaller than in the case of serum cholinesterase, but beyond a certain value of n increases rapidly. If the 'unspecific' forces of attraction between a —CH_2— group and a protein molecule are assumed to be constant, the disturbance probably results from the ionic groups terminating the paraffin chain. Assuming for the time being that the presence of the second quaternary group might be responsible, it can be expected that monoacidic quaternary ammonium salts would not be subject to such an interference and therefore should give a linear plot of $\log K_i$ versus n for both enzymes. This was indeed confirmed by the experimental results shown in Fig. 2. The straight line for serum cholinesterase is almost identical with the corresponding curve in Fig. 1, indicating that the second ammonium group in a methonium compound does not materially contribute to the forces binding these inhibitors to pseudo cholinesterase. The curve for true cholinesterase in Fig. 2 represents measurements at 23° and from its slope $\Delta\Delta F$ is found to be about 300 cal./—CH_2— group.

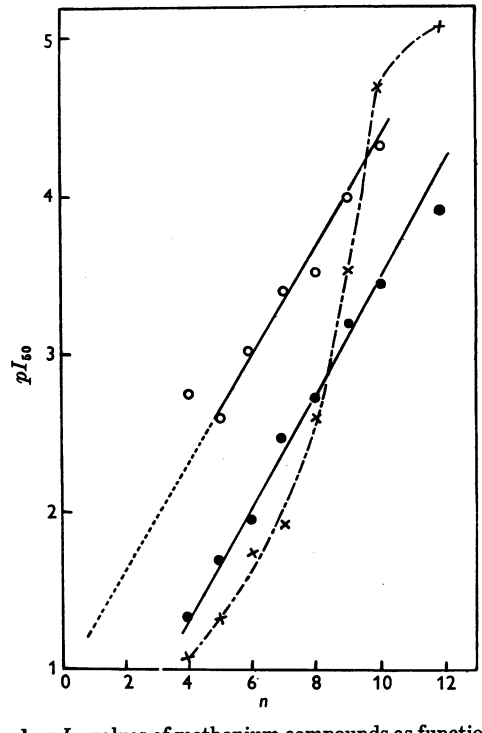

Fig. 1. pI_{50} values of methonium compounds as function of n, the number of carbon atoms between the terminal nitrogen atoms. ○—○, plasma cholinesterase at 37° + acetylcholine, 4×10^{-3} M; ●—●, same enzyme + acetylcholine, 6×10^{-2} M; ×—×, eel esterase at 37° + acetylcholine, 4×10^{-3} M.

Extrapolation of the straight lines in Figs. 1 and 2 to $n=1$ gives a 'theoretical' value of ${}^1I_{50}$, from which ${}^1K_i$ can be calculated using equation (2), if K_m is known. It should be noted that the value for tetramethylammonium, derived in this way, differs from the experimental figure. However, for small values of n, the interaction between an aliphatic carbon and the protein is probably influenced by the neighbouring electrically charged groups. The Michaelis–Menten constant for the system serum cholinesterase-ACh was determined by the method of Dixon (1953). For 'true' cholinesterase, instead of K_m the value of K_1 (Wilson & Bergmann, 1950*b*) was used, although it has been shown in the meantime that the derivation of this constant was based on the incorrect assumption that the anionic site does not undergo any change within the experimental pH range (Bergmann & Shimoni, 1952). Since in tetramethylammonium the aliphatic 'side chain' has been eliminated, ${}^1K_i$ measures the equilibrium for the electrostatic combination of this ion with the anionic site. According to Hammett (1940) the coulombic energy can be calculated from equation 5

$$\Delta F_1 = -RT\log {}^1K_i = \frac{e_1 \times e_2}{D \times r}, \qquad (5)$$

where e_1 and e_2 refer to the charge of inhibitor and enzyme respectively. For the dielectric constant D we use the value 30, as proposed by Pressman, Grossberg, Pence & Pauling (1946). Since $e_1=1$, we can now derive values for the interionic distance r, when $e_2=1$, 2, etc. As shown in Table 3, we obtain with serum cholinesterase $r=5{\cdot}3$ Å, for $e_2=1$ and 10·6 for $e_2=2$. Since the radius of the tetramethylammonium ion is about 4 Å and the C—O bond length of a carboxylate ion 1·4 Å, the distance of closest approach must be of the order of 5·5 Å. For true cholinesterase we find $r=2{\cdot}75$ Å if $e_2=1$. This value evidently is too small. The correct distance $r=5{\cdot}5$ Å is obtained for $e_2=2$. This result suggests not only that two negative sites must be present in the neighbourhood of the esteratic site of true cholinesterase, but also that they must be located close to each other so as to enable them to exert their attractive force on a single positive ion simultaneously.

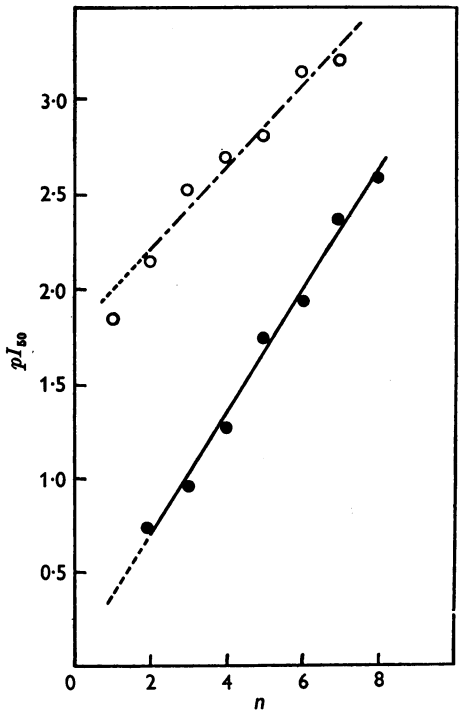

Fig. 2. pI_{50} values of n-alkyltrimethylammonium salts as function of n, the number of carbon atoms in the n-alkyl group: ○, eel esterase at 23° + acetylcholine, 4×10^{-3} M; ●, plasma cholinesterase at 37° + acetylcholine, 6×10^{-3} M.

Table 3. *Free energies, interionic distances and number of anionic sites*

The calculations for this table are based on the formula

$$\Delta F_{\text{molar}} = \frac{Ne_1 \times e_2}{D \times r} = -2{\cdot}3\,RT\log K,$$

where ΔF denotes the free energy change of the process under consideration and K its equilibrium constant; N = Avogadro's number = $6{\cdot}03\times10^{23}$; e_2 = the electronic charge of the active surface of the enzyme and e_1 = the charge of substrate or inhibitor respectively. These charges are expressed as multiples of $4{\cdot}8\times10^{-10}$ e.s.u. The dielectric constant D of the medium is taken as 30 according to Pressman *et al.* (1946); r, the distance of closest approach of the oppositely charged particles, is obtained in cm., but shown after conversion to Å.

	Constant used	ΔF molar (cal.)	$\frac{e_2}{4{\cdot}8\times10^{-10}}$	r(Å)
'Pseudo' cholinesterase	$K_m=5{\cdot}56\times10^{-3}$	3200	—	—
	${}^1K_i=3{\cdot}6\times10^{-2}$*	2030	1	5·3
			2	10·6
'True' cholinesterase	$K_1=2{\cdot}6\times10^{-4}$†	4850	—	—
	$K_2=3{\cdot}2\times10^{-2}$†	2020	—	—
	${}^1K_i=6{\cdot}3\times10^{-4}$*	4350	1	2·75
			2	5·5

* Extrapolated value for tetramethylammonium.
† Taken from the paper of Wilson & Bergmann (1950*b*). See Discussion.

If this conclusion is correct, then monoacidic quaternary ammonium bases produce effective inhibition of true cholinesterase already when only one inhibitor molecule is bound by the enzyme. The number n in the enzyme–inhibitor complex EI_n can be measured in the following way: if in equation 1 we replace I by I^n, suitable transformation leads to expression 6

$$\log (v_0/v_i - 1) = n \log I + C, \qquad (6)$$

where $$C = \log \frac{K_m}{K_i(K_m + S)}.$$

A plot of $\log (v_0/v_i - 1)$ versus $\log I$ should give a straight line, the slope of which yields n directly. As shown in Fig. 3, for the system eel esterase–tetramethylammonium n is about 1, regardless of whether the substrate is ACh or diacetin.

Since our results on the inhibition of serum cholinesterase by methonium compounds differ from those of Paton & Zaimis (1949), we have also carried out a few experiments with the substrate used by these authors, viz. benzoylcholine. The I_{50} values as shown in Table 2 reveal the same trend as is observed for the corresponding concentration of ACh. However, owing to the smaller K_m value for the system benzoylcholine–serum cholinesterase, i.e. $2{\cdot}5 \times 10^{-3}$, the inhibitory effect is much smaller than in the case of ACh as substrate.

DISCUSSION

The negative charge in the active surface of true cholinesterase has been previously determined. Adams & Whittaker (1950) derived their figure of $e_2 = 1$ from a comparison of the K_i values of choline for the two cholinesterases. The authors assumed $e_2 = 0$ for the pseudo enzyme and used the difference of $\log K_i^{\text{true}} - \log K_i^{\text{pseudo}}$ to eliminate all other 'unspecific' binding forces and to single out the coulombic energy of attraction for the negative site of true cholinesterase. In the light of our present results, however, they measured the charge difference between the two enzymes. Since we now have derived the value of $e_2 = 1$ for serum cholinesterase, the calculation of Adams & Whittaker leads logically to $e_2 = 2$ for the true enzyme.

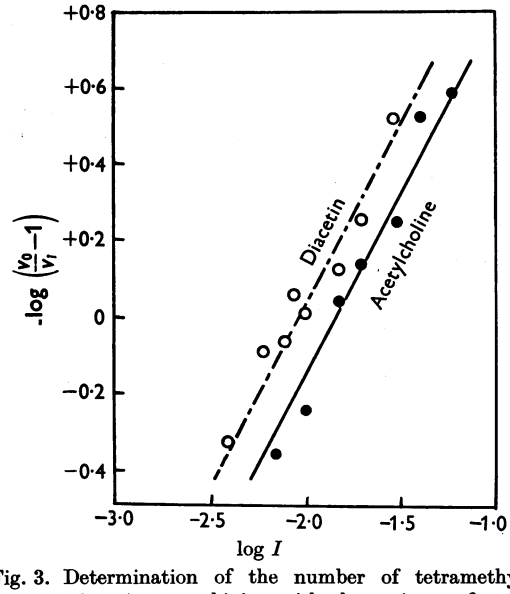

Fig. 3. Determination of the number of tetramethylammonium ions combining with the active surface of true cholinesterase, according to equation 6. Acetylcholine, 4×10^{-3} M; diacetin, $4{\cdot}3 \times 10^{-1}$ M.

It is more difficult to recognize the error underlying the determination of the negative charge in true cholinesterase by Wilson & Bergmann (1950*a*), who also found $e_2 = 1$. These authors compared dimethylaminoethyl acetate at pH 6, where the ester exists almost completely in the ammonium ion form, and at pH 10, where practically only the free base is present in solution. The coulombic energy of attraction was derived from the difference of the $\log K_m$ values at pH's 6 and 10, assuming that at any pH the rate of hydrolysis of the charged form of the ester is a constant fraction of the rate for ACh. It was believed that the protein affinity changes in exactly the same way for both substrates, and that the anionic site does not undergo any changes within the experimental range. The incorrectness of this assumption has been demonstrated subsequently (Bergmann & Shimoni, 1952).

In an earlier paper (Bergmann, Wilson & Nachmansohn, 1950) it was considered that the 'combination of tetramethylammonium with one anionic site should have practically no effect on the reaction velocity, since it essentially replaces normal enzyme molecules by others which have about half the substrate affinity. Therefore, such a type of inhibitor must combine with both anionic sites and form an EI_2 compound in order to prevent the enzymic reaction.' It is now recognized that the spatial arrangement of the two anionic sites in true cholinesterase enables them to combine simultaneously with one and the same inhibitor molecule. Therefore the EI complex is effective as such. However, formation of an EI_2 complex at sufficiently high inhibitor concentrations is thereby not excluded.

The essential difference between the two types of cholinesterase has now been established to lie in the number of negative charges in the neighbourhood of the esteratic site. Experiments, to be published soon, on the pH dependence of the hydrolysis of various substrates show that the esteratic sites of these enzymes are identical in all essential properties. The present results support the previous claim (Bergmann *et al.* 1950) that the formation of an ES_2 compound in the system true cholinesterase–ACh is possible only because of the presence of two negative charges. In addition, it is now

recognized that these charges can approach each other to such a degree as to interact simultaneously with a single quaternary ammonium ion. The complex of three charged particles thus formed is probably arranged in such a way that their centres lie on a straight line and that the potential energy is at its minimum (Fuoss & Kraus, 1933). It might be argued that this description of the spatial arrangement of two negative sites with one positive ion leads to the alternative possibility that one anionic site combines with two positive particles. However, in view of the great difference in size of a carboxylate and a tetramethylammonium ion, the former is much less able to accommodate two units of the latter.

The distance between the two negative charges, which are most probably represented by two carboxyl groups, is, however, not fixed and therefore two positive ions can also be accommodated. Thus, when an ES_2 compound is formed, the two ester molecules are bound each by one anionic site, but compete for the single esteratic site. The two equilibrium constants, which were determined previously (Wilson & Bergmann, 1950*b*), viz. K_1 for the formation of the ES and K_2 of the ES_2 complex, enable us to calculate the difference in binding energy involved in these two cases. It is seen from Table 3 that the binding energy for the second substrate molecule is less than half the value for the first one. In addition, the figure for $\Delta F_2 = 2020$ cal. is practically identical with the value derived from ${}^1K_i$ for serum cholinesterase, viz. 2030 cal., indicating that in the ES_2 complex each cationic ester molecule is bound to one anionic site only. In analogy, since for true cholinesterase we find ΔF (tetramethylammonium) = 4350 cal., it can be concluded that in the ES complex the single substrate molecule is bound simultaneously to both negative sites. This explains the much higher affinity of ACh to true than to pseudo cholinesterase in a qualitative way. However, a quantitative description of the active surfaces will become possible only when all their constants, including the dissociation constants of the anionic sites, have been determined accurately.

Interesting conclusions can be drawn from the I_{50} values of methonium compounds. The single negative charge of serum cholinesterase can accommodate only one positive group. The second cationic end-group does not participate in the binding of the inhibitor, as shown by the closely similar values for ions of equal chain length having one or two quaternary ammonium groups (cf. Figs. 1 and 2). Surprisingly, the second end-group does not even contribute appreciably to the sum of the van der Waals's forces. This is probably due to efficient competition of negative ions in the medium in the absence of a second negative site in the active surface.

True cholinesterase, on the other hand, can accommodate both cationic groups of a methonium compound. However, due to the spatial proximity of the anionic sites to each other, the inhibitor must be 'bent' in order to fit the active surface. 'Bending' becomes much easier, as the chain length increases, since with short chains considerable deviation from the normal bond angles would be involved. Therefore the short molecules resist this process of accommodation to the spatial requirements of the active surface, and show less affinity towards true than towards pseudo cholinesterase. Nevertheless, for true cholinesterase, the electrostatic attraction between the second pair of opposite charges interferes with the coulombic forces of the first pair and thus lowers the inhibitory effect of the shorter members of the methonium series (C_1–C_7) below the I_{50} values of the corresponding monoacidic quaternary ammonium salts.

The S-shaped log S/activity curve of serum cholinesterase can now be explained as due to the presence of a single anionic site, which permits combination with only one cationic ester molecule. The bell-shaped curve for true cholinesterase is related to the two negative charges in the active surface. However, substrates have been found which produce bell-shaped curves even in the absence of any electrostatic forces (Bergmann & Shimoni, 1953). This problem will be dealt with in a future publication.

In the application of these results to *in vivo* effects of methonium compounds we are handicapped by the lack of information on the composition and concentration of the physiological substrate of various cholinesterases. First, the possibility must be considered, that in tissues a mixture of choline esters is present, the composition of which may vary from place to place (Banister, Whittaker & Wijesundera, 1953). Likewise, no reliable information is as yet available on the concentration of substrate or substrates, which is in equilibrium with these enzymes *in vivo*. Therefore, only qualitative conclusions about pharmacological effects can be drawn from *in vitro* measurements. We may, however, predict that all members of the methonium series should be active against both types of cholinesterases in qualitatively the same way. This has been demonstrated by us, e.g. for hexamethonium, which under suitable experimental conditions exhibits a clear-cut blocking effect on the neuromuscular junction, similar to that of decamethonium (unpublished results). It is therefore concluded that the localization of a specific pharmacological effect upon application of 'therapeutic' doses of methonium compounds is due to quantitative differences in the affinities of these inhibitors to the different cholinesterases and thus follows the distribution of these enzymes in the tissues. On the

other hand, the ratio of activity of, for example, hexa- to deca-methonium on the cholinesterases of a given organ may serve as indication of the type of enzyme predominating in this organ. A simple method for the identification of different types of cholinesterases in various tissues can be based on these observations, as will be shown in a subsequent paper.

Application of the method developed here to the measurement of electric charges in the active centre of other enzymes is now being investigated.

SUMMARY

1. A plot of $\log I_{50}$ (I_{50} = concentration of inhibitor, producing 50% inhibition) versus n, the number of carbon atoms between the terminal nitrogens of alkane bistrimethylammonium salts, gives a straight line for serum cholinesterase and a sigmoid curve for eel esterase.

2. The same plot for n-alkyltrimethylammonium salts gives straight lines for both enzymes. The slope of these lines permits calculation of the free energy of the transfer of one —CH_2— group from the solution to the enzyme surface.

3. The extrapolated value of the inhibitor constant for tetramethylammonium makes possible the calculation of the energy of coulombic attraction between this ion and the negative charge in the active surface. From this magnitude the distance of the closest approach can be derived when the charge at the enzyme is 1, 2, 3, etc. unit charges.

4. In this way it is found that serum cholinesterase contains a single, and true cholinesterase two, anionic sites. The latter must be located near to each other and be sufficiently 'elastic' to allow simultaneous interaction with a single positive ion.

5. The possibility is discussed that one molecule of either acetylcholine or inhibitors can be attached simultaneously to both anionic sites of true cholinesterase, whereas when two molecules of substrate are bound to one enzymically active site, each ester molecule combines with one anionic site only.

6. An explanation is forwarded for the different substrate concentration/activity curves of the two cholinesterases.

The authors wish to thank Professor C. Chagas, Department of Physiology, University of Brazil, Rio de Janeiro, and Professor F. Feigl, for the gift of the electric organ of *Electrophorus electricus*. Our thanks are also due to Dr H. J. Barber, of May and Baker Ltd., Dagenham, Essex; to Dr Aeschlimann of Hofmann–La Roche, Nutley, N. J.; to Messrs Allen and Hanburys Ltd., Manchester, England, and to British Industrial Solvents Ltd., London, England, for the materials supplied. This paper forms part of a Ph.D. thesis, submitted by R. Segal to the Faculty of Science of the Hebrew University, Jerusalem.

REFERENCES

Adams, D. H. & Whittaker, V. P. (1950). *Biochim. biophys. Acta*, **4**, 543.

Banister, J., Whittaker, V. P. & Wijesundera, S. (1953). *J. Physiol.* **121**, 55.

Bergmann, F. & Shimoni, E. (1952). *Biochim. biophys. Acta*, **9**, 473.

Bergmann, F. & Shimoni, E. (1953). *Biochem. J.* **55**, 50.

Bergmann, F., Wilson, I. B. & Nachmansohn, D. (1950). *Biochim. biophys. Acta*, **6**, 217.

Bergmann, F. & Wurzel, M. (1954). *Biochim. biophys. Acta*, **13**, 251.

Dixon, M. (1953). *Biochem. J.* **55**, 170.

Edsall, J. T. (1947). *Advanc. Protein Chem.* **3**, 383 (esp. 456).

Fuoss, R. M. & Kraus, C. A. (1933). *J. Amer. chem. Soc.* **55**, 2387.

Hammett, L. P. (1940). *Physical Organic Chemistry*. New York and London: McGraw-Hill.

Paton, W. D. M. & Zaimis, E. J. (1949). *Brit. J. Pharmacol.* **4**, 381.

Pressman, D., Grossberg, A. L., Pence, L. H. & Pauling, L. (1946). *J. Amer. chem. Soc.* **68**, 250.

Rothenberg, M. A. & Nachmansohn, D. (1947). *J. biol. Chem.* **168**, 223.

Wilson, I. B. & Bergmann, F. (1950*a*). *J. biol. Chem.* **185**, 479.

Wilson, I. B. & Bergmann, F. (1950*b*). *J. biol. Chem.* **186**, 683.