THE CHOLINESTERASES OF HUMAN BLOOD

II. THE FORCES ACTING BETWEEN ENZYME AND SUBSTRATE

by

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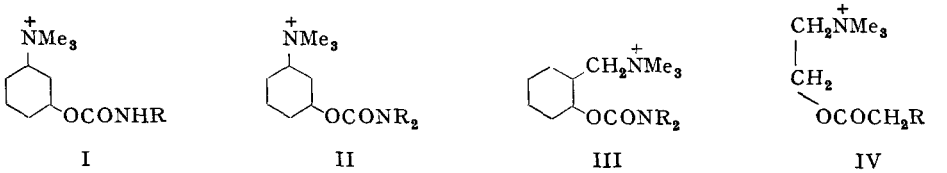
Our investigations into the specificity of the cholinesterases of human blood[1, 2, 3] have recently shown that their specificity is primarily determined not by the chemical nature of the substrate molecule, but by its shape. Thus the erythrocyte cholinesterase is not merely capable of hydrolysing two or three closely related choline esters; it will hydrolyse many acetate (and propionate) esters, the rate of hydrolysis of non-choline acetates markedly increasing through the series *n*-butyl, *iso*-amyl, and 3:3-dimethyl butyl acetate. The last of these substances, the nearest approach to the acetyl choline configuration possible in the aliphatic series, is split more rapidly than any acetate ester other than acetyl choline itself. A similar situation exists with the plasma enzyme, butyryl choline and the aliphatic butyrates bearing much the same relation to it as do acetates to the erythrocyte enzyme.

The importance of molecular shape for cholinesterase activity is also illustrated by the numerous specific inhibitors of this group of enzymes. The miotin[4, 5] (Fig. 1, formula I), prostigmine[5] (II) and NUTLEY[6] (III) series of competitive inhibitors most likely owe their inhibitory effect to a close analogy in configuration to choline esters (IV).



I II III IV

Fig. 1

Presumably the inhibitory power of such substances is due to a strong affinity for the enzyme occasioned by their similarity in shape to the substrate molecules, combined with a stability not possessed by carboxylic esters when absorbed onto the active centres.

Such facts, and similar properties of other enzymes[7, 8] clearly demand the conception, first introduced into biochemistry by FISCHER[9] in his famous "lock and key" simile, of a complementary relation between the shape of the substrate or competitive inhibitor molecule on the one hand, and the active centres of the enzyme on the other. The limitation of this hypothesis is that it leaves unsettled the nature of the forces operating between enzyme and substrate and leading to the disruption of the latter.

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In recent years the idea has become current that substrates may, in many cases, be anchored to an enzyme by chemical combination with certain reactive groups in the enzyme surface. The use of certain reagents has shown that many enzymes contain –SH groups whose integrity is essential for their action and which are closely associated with the active centres. In other cases, tyrosine –OH groups or $-NH_2$ groups may play an analogous rôle. Reactive groups such as these may combine with appropriate groups in the substrate molecule to form an enzyme substrate complex; thus the formation of a $>C=N-$ link has been postulated between pyruvate and carboxylase[35]. In dehydrogenases on the other hand, essential –SH groups may be involved in H^+ and electron transfer from a substrate otherwise anchored to the enzyme surface[11]. Essential –SH groups have been shown to be present in certain hydrolytic enzymes such as lipase and cholinesterase, but it is unlikely that these can be directly concerned in linking the substrate to the enzyme by reacting with the former. Evidence has indeed been recently presented[12] which suggests that essential –SH groups may play a somewhat different rôle in oxidative and in hydrolytic enzymes. SINGER has shown that a given concentration of the non-competitive inhibitor *p*-chlormercuribenzoic acid produces essentially the same inhibition of D-aminoacid oxidase, whichever of a number of aminoacids is used as substrate; in the case of pancreatic lipase, however, the percentage inhibition produced by a given concentration of mercurial varies widely with the substrate used; the variation can be correlated with the size of the substrate molecule, being least with the substrates of smallest molecular size. SINGER points out that this result could be accounted for if it is assumed that the –SH groups are identical with the active centres of the enzyme in the case of the oxidizing enzyme, but not in the case of the hydrolytic enzyme. In the latter they serve to anchor inhibitor molecules but not substrate molecules; the inhibitor then exerts a steric interference with the approach of the substrate to the otherwise unaffected active centres which will be greatest with the bulkiest substrate molecules.

Covalent bonding between substrate and enzyme is by no means the only physical force which could operate between them. Hydrogen bonding is a kind of weak chemical link which has accounted for a large number of chemical phenomena for which no explanation can be offered in terms of classical chemical theory, and a number of types of electrical interaction between molecules are also recognized, ranging from the Coulomb interaction of ions of opposite sign which constitutes the main valency force in crystalline salts to the undirected and non-specific Van der Waals or dispersion forces which are responsible for the aggregation of molecules in the liquid and solid states. It has been pointed out by PAULING[13] that over the region of contact of complementary molecular configurations Van der Waals forces may well be considerable.

The various types of electrical interaction of molecules are as follows[14]:

Coulomb interaction (ion-ion, electrovalency, salt link)
Ion-dipole
Ion-induced dipole
Dipole-dipole
Dipole-induced dipole ("induction force")
Induced dipole-induced dipole ("dispersion force").

Usually only one or two of these forces contributes significantly to the total force of attraction in any particular system.

Little attempt has been made to apply the theory of intermolecular forces to the

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problem of enzyme-substrate interaction. A somewhat analogous problem has however been studied, namely the combination of haptenic groups with antibodies. LANDSTEINER[15] was one of the first to show that antibody formation could be successfully evoked by synthetic antigens consisting of a protein to which had been coupled a haptenic group consisting of an arsonic acid or other organic molecule. From a study of cross reactions between the antibody and other antigens containing related haptenic groups it has been established that the specificity of the antigen-antibody reaction is determined not by any specific chemical grouping but by the molecular shape of the haptenic group. A somewhat more convenient method of studying antibody specificity is to make use of the ability of the free haptene molecule and its derivatives to inhibit, in varying degrees, the formation of antibody-antigen precipitate. PAULING and his associates have carried out a large number of experiments of this kind, and have discussed in some detail the results obtained in terms of VAN DER WAALS and other intermolecular forces[16, 17, 18].

The treatment adopted by PAULING *et al.* is obviously applicable to enzymes[19], at any rate where there is evidence, as here, that specificity is related to molecular shape rather than to specific chemical groupings. The purpose of this paper is to give an account of some preliminary results in this direction together with a discussion of some of the theoretical assumptions and difficulties involved.

METHODS

A. THEORETICAL

Relation between affinity constant and energy of attraction. Theoretical evaluation of the energy of attraction between a substrate or inhibitor molecule and the active centres of the enzyme is impossible, because there is, as yet, no detailed knowledge available of the molecular structure of the active centres. It may however be possible, in certain cases, to adopt a relative treatment. Consider a pair of molecules A–B and A′–B, where the groups A and A′ are small relative to the rest of the molecule B and do not differ appreciably in size. Many of the properties of molecules, such as polarizability, are additive to a fairly high degree of approximation. We may, therefore, consider the energy of attraction, if due to any of the intermolecular forces listed in the introduction, as made up of two terms, $E_1 + E_2$, (where E_1 relates to B and E_2 to A) the corresponding quantities for the second molecule being $E_1 + E_2'$. The difference in the energy of attraction, ΔE, of the two molecules is thus

$$\Delta E = E_1 + E_2 - (E_1 + E_2') = E_2 - E_2'$$

The affinity of an inhibitor or substrate (A) for an enzyme (E) is measured by the affinity constant, which will be denoted in this paper by the symbol K_a. It is the equilibrium constant of the reaction,

$$\mathrm{E} + \mathrm{A} \rightleftharpoons \mathrm{EA}$$

and is the reciprocal of the dissociation constant of the enzyme-substrate complex or Michaelis constant, as usually defined. The standard free energy change ΔF^* of the above reaction (the free energy of activation) is related to the affinity constant by the usual equation

$$\Delta F^* = -RT \ln K_a = -2.3\, RT \log_{10} K_a$$

where R is the gas constant and T the absolute temperature.

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It is clear that we are not, in general, entitled to equate $-\Delta F^*$ with the energy of attraction $E_1 + E_2$ considered above. Apart from kinetic energy considerations, adsorption of a substrate onto the active centres will involve distortion or activation of the substrate molecule, the energy required being provided by the energy of attraction. With inhibitors, on the other hand, deformation may well be small or absent and the free energy of activation may be more closely related to the potential energy of attraction.

If we now consider the *difference* in the free energy of activation of molecules closely similar in size and deformation energy, the additional energy terms will cancel out and the relation

$$\Delta F^* - \Delta F^{*\prime} = -RT \ln K_a/K_a' = -(E_2 - E_2') \qquad (1)$$

would be expected to hold. Since, as will be shown, E_2 and E'_2 may in certain circumstances be evaluated theoretically, we can obtain a theoretical value for the *ratio* of the affinity constants of the two substances (affinity ratio) which depends solely on the electrical properties of A and A′ and which can be compared with experiment.

The approach outlined above may also be useful in studying the relation of two distinct but closely similar enzymes. Consider two enzyme inhibitor complexes EI and E′I formed by the combination of an inhibitor I with two enzymes E and E′. If K_a and K_a' are the affinities of the inhibitor for the enzymes, the difference in the free energy of attraction will be

$$\Delta E = 2.3\ RT \log K_a/K'_a \qquad (2)$$

Physical models of the active centres of the enzymes may be tested by evaluating ΔE and K_a/K_a' theoretically and again comparing the result with experiment.

Evaluation of affinity ratio: theory of competition experiments. The experimental determination of affinity constants is somewhat tedious, involving the measurement of the initial velocity of the enzyme reaction at a number of different substrate concentrations. We have accordingly devised a more convenient, if less accurate method, based on an extension of the Michaelis kinetics, for obtaining the affinity ratio for two substrates directly from competition experiments in which the rate of hydrolysis of the mixed substrates, in the presence of a single enzyme, is compared with the rate of hydrolysis of each substrate measured separately. The theory of the method is as follows. Consider an enzyme the concentration of whose active centres is initially E, and two substrates initially present in concentrations A and B. Let the concentration of enzyme substrate complexes be EA and EB, and let the affinity constants of EA and EB be K_a and K_a'. Then

$$K_a = \frac{\mathrm{EA}}{(\mathrm{E} - \mathrm{EA} - \mathrm{EB})\,\mathrm{A}}$$

$$K_a' = \frac{\mathrm{EB}}{(\mathrm{E} - \mathrm{EA} - \mathrm{EB})\,\mathrm{B}}$$

Let the combined rate of hydrolysis when both esters are present be v; then

$$v = K\mathrm{EA} + K'\mathrm{EB}$$

where K and K' are the rate constants for the breakdown of EA and EB. Eliminating EA and EB from these equations, we have

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$$v = \frac{K_a KEA + K_a'K'EB}{K_aA + K_a'B + 1}$$

if A = B = S,

$$v = \frac{K_aKE + K_a'K'E}{K_a + K_a' + 1/S}$$

$$\text{and } \lim_{S \to \infty} v = V_m = \frac{K_a KE + K_a'K'E}{K_a + K_a'} = \frac{K_aV + K_a'V'}{K_a + K_a'}$$

where V and V' are the limiting velocities of A and B determined separately. Then the affinity ratio is

$$K_a/K_a' = \frac{V_m - V'}{V - V_m} \quad (3)$$

The use of this equation to determine the affinity ratio assumes that the kinetic behaviour of each substrate is reasonably well represented by the simple MICHAELIS theory and that the concentrations of substrates are equal and are on the plateaux of their respective Michaelis curves. It is very sensitive to errors in the values of V, V_m and V'. Reasonable agreement between experiments ($\simeq$ 10%) can be obtained, however, provided that $V - V_m$ and $V_m - V'$ are not too small.

B. EXPERIMENTAL

Purified preparations of human erythrocyte and plasma cholinesterase were obtained as described in previous papers[2, 20, 3]. Enzyme activity was measured by the Warburg technique[21]. Aliphatic substrates were prepared by standard methods and carefully purified by distillation *in vacuo* before use; choline and choline esters were obtained commercially.

RESULTS

A. THE "NITROGEN ATTRACTING GROUP" IN THE ERYTHROCYTE CHOLINESTERASE

The first example we have chosen of applications of the theoretical suggestions made in the previous section is an analysis of certain differences in the kinetic behaviour of the plasma and erythrocyte enzymes, which points to the existence of a "nitrogen attracting group" in the active centres of the latter enzyme which increases its affinity for positively charged ions relative to the plasma enzyme.

It is well known that acetyl choline exhibits, in concentrations above about 1 mM, marked auto-inhibition of the erythrocyte enzyme. Such humped initial velocity-substrate concentration curves are not found with the plasma enzyme, even with acetylcholine, though slightly humped curves intermediate between the fully humped and classical Michaelis form have been observed[22] with certain substrates.

Initial velocity-concentration curves with marked auto-inhibition were first studied by MURRAY AND HALDANE[23, 24] using liver esterases. They can be accounted for theoretically by considering the mechanism

$$E \text{ (Enzyme)} + S \text{ (Substrate)} \overset{K_1}{\rightleftharpoons} ES \overset{K}{\rightarrow} E + P \text{ (Products)}$$

$$ES + S \overset{K_2}{\rightleftharpoons} ES_2 \text{ (inactive second enzyme substrate complex)}$$

where K_1 and K_2 are dissociation constants and K is a velocity constant.

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The possibility of ES_2 formation at first sight seems puzzling, but there seems no reason to doubt the applicability to the cholinesterases of MURRAY's suggestion[23] that in ES_2, one ester molecule is attached to the enzyme by the acyl group and the other by the alcohol group thus obviating the 'strain' which may result from both parts of a single substrate molecule being simultaneously attached to the active centre[10]. The occurrence of a MURRAY-HALDANE curve with acetyl choline and the erythrocyte enzyme suggests that here at least the ES_2 configuration is unusually stable, and that acetyl choline can attach itself strongly to the enzyme by its choline group perhaps because of an electrostatic attraction between its positively charged nitrogen and some sort of 'nitrogen attracting group' associated with the alcohol part of the active centre of the enzyme. The absence of autoinhibition with other substrates in the presence of the erythrocyte enzyme is comprehensible; substrates lacking a positively charged nitrogen (as with the aliphatic substrates) or departing from the acetyl choline configuration (as in acetyl-β-methyl choline), would be expected to show a weakened attraction between their alcohol groups and the corresponding portion of the active centre.

The absence of MURRAY-HALDANE curves in the case of the plasma enzyme, even with such substrates as acetyl or butyryl choline, is more difficult to interpret, especially as our specificity studies with this enzyme[3] show that as with the erythrocyte enzyme, substrate specificity is determined by both parts of the ester molecule, suggesting two point attachment in contradiction to ZELLER AND BISSEGGER's conception[10]. The most likely explanation is that ES_2 formation occurs to a much smaller or negligible extent because of the non-existence or considerably smaller effect of the 'nitrogen attracting group'. We have attempted to investigate the nature of this 'nitrogen attracting group' by two methods: a) by measurements of the affinity of choline for the two enzymes; b) by a comparison of the affinity of the uncharged carbon analogue of acetyl choline (3:3-dimethyl-*n*-butyl acetate) with that of acetyl choline itself.

The affinity constant of choline. Fig. 2 shows the percentage inhibition of erythrocyte and plasma cholinesterase produced by different concentrations of choline chloride in the presence of 0.012 M acetyl choline. The ordinates are the reciprocals of the concen-

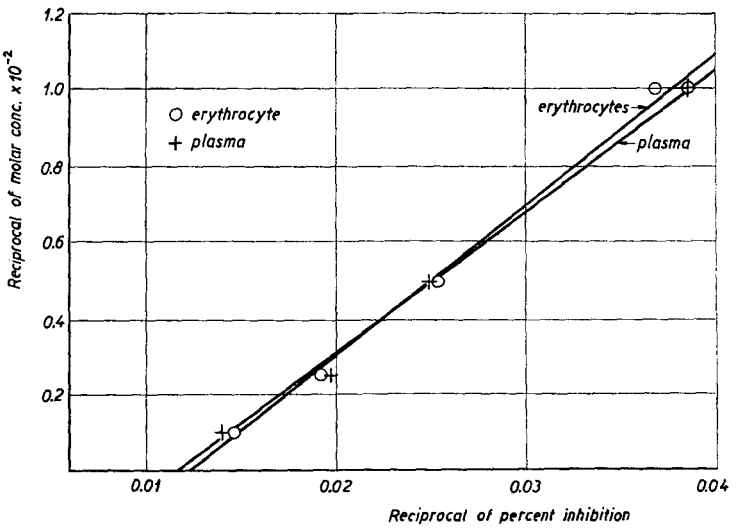

Fig. 2. Inhibition of cholinesterases of human blood by choline (substrate, 0.012 M acetyl choline)

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trations used, and the abscissae, the reciprocals of the percentage inhibition produced by these concentrations. The lines are the lines of nearest fit calculated in the usual way by the method of least squares. In this way the most probable value for the I_{50} concentration (concentration producing 50% inhibition) has been obtained; it is for the erythrocyte enzyme 0.0334 M and for the plasma enzyme 0.0326 M. To calculate the affinity constants we have had to assume values for the dissociation constants of the ACh-enzyme complexes. For the plasma enzyme the average of the values given by GLICK[25] and WRIGHT AND SABINE[26, 27] has been used, namely $1.47 \cdot 10^{-3}$ M. The values for the erythrocyte constants present a difficulty in that they show a well established variation with salt concentration. Thus WRIGHT AND SABINE[27] have tabulated the ACh dissociation constants of human r.b.c. and mouse brain cholinesterase derived from their own, and other workers' data alongside the $Na^+ + K^+$ molar concentration used in each determination (Table II of their paper). The value of the first dissociation constant K_{s_1} corresponding to the highest salt concentration differs by a factor of 10 from that corresponding to the lowest. We have accordingly obtained the value of K_{s_1} corresponding to our salt concentration (0.023 M) by interpolation on the straight line of nearest fit for WRIGHT AND SABINE's collected data, assuming a linear relation between K_{s_1}, and salt concentration and excluding the point (0.145, 1.2) which is very aberrant. This gives $K_{s_1} = 0.44 \cdot 10^{-4}$ M. We are now in a position to calculate the affinity constants K_a and K_a' for choline and the plasma and erythrocyte enzymes. These are the reciprocals of the enzyme-inhibitor dissociation constants and are given by the expressions, for plasma,

$$K_a = \frac{1 + S/K_s}{I_{50}}$$

and for erythrocytes,

$$K_a' = \frac{1/K_{s_1} + S^2/K_{s_2}}{I'_{50}}$$

where S is the substrate concentration, K_s, K_{s_1} and K_{s_2} the appropriate dissociation constants for ACh, and I_{50} and I'_{50} the concentrations of choline producing 50% inhibition of the plasma and erythrocyte enzymes respectively. In our experiments $S = 0.012$ M, so that the term in S^2 can be neglected, whence $K_a' = 8130$, $K_a = 283$ and $K_a'/K_a = 28.8$. The value for plasma is close to that of AUGUSTINSSON[22] for horse serum, 313.

It will thus be seen that although a given concentration of choline under our conditions produces an approximately equal inhibition of both enzymes, its affinity for the erythrocyte is nearly 30 times that for the plasma enzyme. Since it is the strength of attachment at the choline end of the molecule which determines the stability of the ES_2 configuration, this would seem good evidence for the existence of a "nitrogen attracting group" in the erythrocyte enzyme which is larger in its effect than the corresponding group, if, indeed, it exists at all, in the plasma enzyme.

Magnitude of the 'nitrogen attracting group'. We may now attempt to calculate the distance a unit charge would have to be from the charge on the choline ion to account for the difference in the affinity constants of choline. The chief difficulty in any calculation of this kind is the lack of any reliable estimate for the dielectric constant D in the expression for the Coulomb interaction energy of charges e_1 and e_2, e_1e_2/Dr.[28] We may however provisionally accept the value of 30 used by PRESSMAN *et al.*[18] in a similar

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calculation involving the haptene inhibitor constants for "H-acid"-*p*-azo-phenyl-trimethylammonium ion and "H-acid"-*p*-azo-*tert.*-butyl benzene and the "A*p*" horse serum albumin "anti-A*p*"-serum system.

The difference in the energies of attraction, E and E' of choline for the plasma and erythrocyte enzymes can be assumed to be due to an attraction between positively charged nitrogen of the choline molecule and a hypothetical unit negative charge r Ångström units from it which exists in the erythrocyte but not in the plasma enzyme. Applying equation (2),

$$2.3\ RT \log \frac{K_a'}{K_a} = E' - E = Ne^2/Dr$$

whence,

$$r = \frac{Ne^2}{2.3\ DRT \log K_a'/K_a}$$

where primed symbols refer, as before, to the erythrocyte enzyme, and unprimed to the plasma enzyme, N is Avogadro's number and e, the charge on the electron. Accordingly, since $K_a'/K_a = 28.8$, $R = 8.314 \cdot 10^7$ ergs/degree-mol, $T = 311$, $N = 6.03 \cdot 10^{23}$, $D = 30$, $e = 4.8 \cdot 10^{-10}$ esu

$$r = \frac{6.03 \cdot 10^{23} \cdot (4.8 \cdot 10^{-10})^2 \cdot 10^8}{30 \cdot 2.3 \cdot 8.314 \cdot 10^7 \cdot 311 \cdot 1.459}\ \text{Å} = 5.3\ \text{Å}$$

This value for r is significantly close to the nearest distance of approach, 4.9 Å of a positive charge situated at the centre of a trimethylammonium ion and a negative charge situated at the centre of an oxygen atom (*e.g.*, of a COO^- group) in contact with the $\overset{+}{N}Me_3$ group[18].

This treatment does not of course reveal whether the plasma enzyme also possesses an electric charge which is capable of contributing to the total energy of attraction; it shows merely that the erythrocyte has a stronger 'nitrogen attracting group'. We have attempted to decide to what extent an electrostatic attractive force contributes to the affinity of choline esters for the plasma enzyme by comparing the affinity of acetyl choline with that of its carbon analogue, 3:3-dimethyl-*n*-butyl acetate. This compound is almost identical to acetyl choline in shape, size, polarizability and in other relevant properties with the exception that it possesses no positive charge.

Affinity ratio for acetyl choline and its carbon analogue. Using equation (3) we have obtained a mean value of 1.29 for the ratio of the affinities of acetyl choline and its carbon analogue, 3:3-dimethyl butyl acetate, for the plasma enzyme. If these affinities are denoted by K_{ACh} and $K_{C\text{-analogue}}$, e is the charge on the acetyl choline ion, e' the charge of opposite sign on the protein and r' the distance between these charges, from equation (1),

$$E_2 - E_2' = \frac{Nee'}{Dr'} = 2.3\ RT \log K_{ACh}/K_{C\text{-analogue}}$$

whence $e'/r' = 0.68 \cdot 10^{-3}$ esu/cm.

The corresponding potential for the erythrocyte enzyme is

$$\frac{4.8 \cdot 10^{-10}}{5.3 \cdot 10^{-8}} + 0.68 \cdot 10^{-3}\ \text{esu/cm}$$

or $9.9 \cdot 10^{-3}$ esu/cm.

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The nitrogen attracting group in the plasma enzyme, if it exists, would have to exert, therefore, less than 7% of the potential of the corresponding group in the erythrocyte enzyme to account for the observed $K_{ACh}/K_{C\text{-analogue}}$ ratio.

It is not, however, necessary to postulate an electrostatic interaction of the Coulomb type to account for the observed value of the $K_{ACh}/K_{C\text{-analogue}}$ ratio. The positively charged ion will induce a dipole, the magnitude of which depends on the polarizability of the protein, which will interact with the positive charge of the ion to produce an attractive force, even though the active centre of the protein may have been initially neutral. No such attractive force can be engendered by the uncharged carbon analogue and since other kinds of induction or dispersion forces will be approximately equal for the two substrates, the difference in the energy of attraction must, on this theory, be equated with the ion-induced dipole interaction. The magnitude of this energy is given by the expression[14],

$$E = N\alpha e^2/2Dr^4 = 2.3\ RT \log \frac{K_{ACh}}{K_{C\text{-analogue}}}$$

where α is the polarizability of the protein, e the charge on the ion, r the distance between substrate and the active centre of the protein and the other symbols as previously defined.

Now

$$\alpha = \frac{3R_p}{4\pi N}$$

where R_p is the mole refraction. The value for plasma cholinesterase is unknown, but following PAULING AND PRESSMAN[17] we may take it to be approximately equal to 47.2 ml a value derived from that for squash seed globulin. For r the value found for the erythrocyte cholinesterase, 5.3 Å, is assumed. Then,

$$\log \frac{K_{ACh}}{K_{C\text{-analogue}}} = \frac{3R_p e^2}{4\pi \cdot 2Dr^4 \cdot 2.3\ RT}$$

$$= \frac{3 \cdot 47.2 \cdot (4.8 \cdot 10^{-10})^2}{4 \cdot 3.14 \cdot 2 \cdot 30 \cdot (5.3 \cdot 10^{-8})^4 \cdot 2.3 \cdot 8.3 \cdot 10^7 \cdot 311} = 0.0923$$

whence $K_{ACh}/K_{C\text{-analogue}} = 1.24$, a value close to the experimental value, 1.29.

Much uncertainty attaches to certain of the quantities used in this calculation but it does suggest that the observed ratio of affinities of these substrates is not of such an order of magnitude as to require the assumption that a negative nitrogen attracting group exists in the plasma enzyme.

B. THE EFFECT OF CHANGES IN POLARIZABILITY AND DIPOLE MOMENT ON THE AFFINITY OF SUBSTRATES FOR THE CHOLINESTERASES OF HUMAN BLOOD

In the previous section, the absence of marked auto-inhibition as a feature of the initial velocity-concentration curves of plasma cholinesterase and the small difference in magnitude between the affinity of the charged acetyl choline ion and its uncharged carbon analogue for this enzyme have led to the conclusion that whatever the nature of the forces binding enzyme and substrate it is unlikely that a negative attracting

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charge is associated with the active centres. This suggests that in the case of aliphatic substrates and plasma cholinesterase, the attractive force between enzyme and substrate may be due primarily to the interaction of dipoles. The most important contribution to such forces will be made by the so-called dispersion or Van der Waals forces. The magnitude of these forces is determined by the polarizability of the interacting molecular structure, which is in turn proportional to the mol refraction, so that if dispersion forces constitute the principle forces of attraction between the plasma enzyme and its substrate, it should be possible to account for any changes in affinity in terms of changes of polarizability.

We have sought to test this hypothesis by comparing the ratio of the affinities of chloro- and of bromo-acetates with that of the corresponding propionates, with the value calculated from the change in polarizability; *n*-butyl and *iso*-amyl esters were used, and a few experiments were also carried out with the chloroacetate and propionate of 3:3-dimethyl butyl alcohol. The alkyl groups of all these esters contain a chain of 4 carbon atoms corresponding to the –C–C–N–C chain of choline; it was felt that the use of esters further removed than *n*-butyl from the choline configuration might introduce a needless complication. The contact radii[29] of chlorine (1.8 Å) and of bromine (1.95 Å) are very little less than that of a methyl group (2.0 Å) so that changes of affinity due to the purely steric factor of altered acyl group size will be small. On the other hand the mol refraction of aliphatic chlorine (related to the polarizability by the factor $4\pi N/3$) is somewhat greater (6.57 ml) than that of the methyl group (6.34 ml), and that of bromine (9.47 ml) is considerably greater[30]. In fact, as will be seen later, the aliphatic chloroacetates and bromoacetates tested have greater affinities for the plasma enzyme than the corresponding propionates, the increase with bromoacetates being larger than that with chloroacetates. The affinity ratios obtained theoretically show surprisingly good agreement in view of the many approximations made.

In the case of the erythrocyte enzyme, calculations of affinity ratios will be more difficult to make if, as the experiments with choline suggest, there is a negative charge associated with the active centres. Under these circumstances, the contribution made by the dispersion forces will be exceeded by ion-dipole forces which fall off much less rapidly with distance[14]. Further, since the propionyl group is considerably larger than the optimum acyl group (acetyl) for this enzyme, the changes in acyl group size involved in replacing methyl by the slightly smaller halogens may produce a larger effect than with the plasma enzyme. In fact, the affinity ratios obtained with erythrocyte preparations have been found to be somewhat different from those obtained with the plasma enzyme and are not consistent with the values obtained from a consideration of dispersion forces alone.

Affinity ratios for the plasma enzyme. The treatment of PAULING AND PRESSMAN[17] developed for the interaction of haptene inhibitors with specific antibodies, can be adapted as follows. The Van der Waals energy of interaction E in cal/mol of two structures A and B is given approximately by the expression

$$E = \frac{23000}{r_{AB}^6} R_A R_B$$

where R_A, R_B, are the mol refractions in ml of A and B and r_{AB} the distance apart (in Å) of A and B. This equation contains an arbitrary correction for the contribution of short range repulsion to the interaction energy.

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In applying this formula to the interaction of a group A with enzyme E an allowance must be made for the energy of desolvation of the group A and the active centre of the enzyme.

Accordingly the net energy of attraction of A for the enzyme is

$$E_2 = 23000 \left[\frac{R_A R_E}{r^6_{AE}} - \frac{R_A R_{aq}}{r^6_{Aaq}} - \frac{R_E R_{aq}}{r^6_{Eaq}} \right]$$

and the difference in energy of attraction for groups A and A′ is

$$E_2 - E_2' = 23000 \left[\frac{R_A R_E}{r^6_{AE}} - \frac{R_{A'} R_E}{r^6_{A'E}} - \frac{R_A R_{aq}}{r^6_{Aaq}} + \frac{R_{A'} R_{aq}}{r^6_{A'aq}} \right]$$

Assume $r_{AE} \simeq r_{Aaq} = r + 2$, $r_{A'E} \simeq r_{A'aq} = r' + 2$ where r, r' are radii of groups A and A′. Then from equation (1),

$$\log K_a/K_a' = \frac{23000}{2.3\,RT} (R_E - R_{aq}) \left[\frac{R_A}{(r+2)^6} - \frac{R_{A'}}{(r'+2)^6} \right]$$

Assuming PAULING AND PRESSMAN's value for $R_E - R_{aq}$ namely 17.6, the numerical factor becomes 288 at 38° C, *i.e.*

$$\log K_a/K_a' = 288 \left[\frac{R_A}{(r+2)^6} - \frac{R_{A'}}{(r'+2)^6} \right]$$

For chloroacetates and the corresponding propionates A = Cl, A′ = CH_3, R_A = 6.57 ml, $R_{A'}$ = 6.34 ml, r = 1.8 Å, r' = 2.0 Å, whence

$$\log K_{Cl}/K_{CH_3} = 0.17, \quad K_{Cl}/K_{CH_3} = 1.48$$

For bromoacetates and the corresponding propionates,

$$\log K_{Br}/K_{CH_3} = 0.262, \quad K_{Br}/K_{CH_3} = 1.83$$

Experimental values for these ratios, obtained by the method of competition experiments described above for *n*-butyl and *iso*-amyl esters are given in Table I. It will be seen, as required by theory, that (a) the bromoacetate/propionate ratios are greater than the chloroacetate/propionate ratios; (b) the ratios are independent of the alcohol group. Moreover the numerical agreement while not exact, is promising. Supplies of

TABLE I

AFFINITY RATIOS OF HALOACETATES AND CORRESPONDING PROPIONATES

Ester	log K_{Cl}/K_{CH_3}			log K_{Br}/K_{CH_3}		
			Plasma			
butyl	0.12	0.09	0.11	0.25	0.27	0.26
iso-amyl	0.13	0.14	0.11	0.24	0.24	0.21
calcd.		0.17			0.26	
			Erythrocyte			
butyl	0.53	0.60		0.43	0.39	0.37
iso-amyl	0.59	0.56	0.62	0.31	0.31	0.32
calcd.		0.40			0.55	

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3:3-dimethyl butyl alcohol were not sufficient to permit a complete series of results to be obtained, but the log of the chloroacetate/propionate affinity ratio for plasma, (av. 0.12), agreed well with the log ratios for *n*-butyl and *iso*-amyl.

Erythrocyte enzyme. Table I also gives the corresponding values of the ratios for the erythrocyte enzyme. It will be seen that while the ratios obtained are again largely independent of alcohol configuration, both chloroacetates and bromoacetates have a higher affinity for the enzyme relative to the propionates than with the plasma enzyme and replacement of chlorine by bromine has now reduced the affinity of the substrate for the enzyme instead of increasing it.

Accepting the idea of a negative charge in close proximity to the active centres of the erythrocyte enzyme, a theoretical account of the energy of interaction of substrate and enzyme must include not only the Van der Waals energy of interaction already considered, but also ion-permanent dipole and ion-induced dipole forces. To avoid unknown orientation, we may consider a model in which the ion-dipole interaction is maximal, *i.e.*, in which the carbon atom bearing the terminal methyl group or halogen atom is at the nearest distance of approach to a negatively charged oxygen atom in the enzyme and in which the charge is in the projected axis of the dipole; this arrangement was found to be feasible in trials with Fischer-Hirschfelder models. The separation r_a of charge and dipole for the halogen esters is, contact radius of oxygen + contact radius of carbon + average of bond radii of carbon and halogen (3.88 and 3.96 Å. for Cl and Br respectively); the dipole moments (μ) of C–Cl and C–Br are in such a direction as to lead to a net attraction in the orientation considered here and have magnitude 1.5 and 1.4 Debye units respectively[31]. The dipole moment of methyl (μ') is equivalent to that of a C–H bond; its direction is opposite to the C–Cl and C–Br moments. Its separation (r_a') from the centre of the negative charge depends on whether the oxygen is in contact with the carbon atom bearing the methyl group or in contact with the carbon of the methyl group itself; the average of these two separations is 4.47 Å. The magnitude of the C–H moment is, however, so small that its contribution to the energy of interaction will be low.

The expressions for the various components of the energy of interaction, and their numerical values, are given below. It will be seen that the contribution of the ion-induced dipole effect is very small in comparison with the ion-permanent dipole effect. It should perhaps again be emphasized that the contribution of that part of the ester molecule which is common to the substances whose energies of interaction are being compared is assumed to be constant and to cancel out when *differences* of interaction energy are being, as here, considered.

Chloroacetate/propionate affinity ratio

$$\frac{\text{Ion-dipole interaction energy}}{2.3\,RT} = \frac{Ne}{2.3\,DRT}\left(\frac{\mu}{r_a^2} - \frac{\mu'}{r_a'^2}\right)$$

$$= \frac{6\cdot 10^{23}\cdot 4.8\cdot 10^{-10}\cdot 10^{-2}}{2.3\cdot 30\cdot 8.3\cdot 10^{7}\cdot 311}\left[\frac{1.5}{(3.88)^2} + \frac{0.4}{(4.47)^2}\right] = 0.193$$

$$\frac{\text{Ion-induced dipole interaction energy}}{2.3\,RT} = \frac{3e^2}{2.3\cdot 4\,\pi\,DRT}\left(\frac{R_A}{r_a^4} - \frac{R_A'}{r_a'^4}\right)$$

$$= \frac{3\cdot (4.8)^2\cdot 10^{-20}\cdot 10^{14}}{2.3\cdot 4\cdot 3.14\cdot 30\cdot 8}\left[\frac{6.57}{(3.88)^4} - \frac{6.34}{(4.47)^4}\right] = 0.037$$

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$$\frac{\text{Van der Waals interaction energy}}{2.3\ RT}\ \text{(as before)} = 0.172$$

Total $= \log K_{Cl}/K_{CH_3} = \underline{0.402}$

The corresponding figures for bromoacetate/propionate are

$$\log K_{Br}/K_{CH_3} = 0.177 + 0.115 + 0.262 = \underline{0.554}$$

A comparison with the experimental figures in Table I shows that these figures differ both in magnitude and trend from those found experimentally. It may be that purely steric factors not allowed for on the above theoretical model contribute to the final result. In any case it seems difficult to account for the observed fall in the affinity ratio with bromine as although its dipole moment is less than chlorine and its radius is greater, the difference in either of these quantities is much less than the experimental difference in the affinity ratio.

Rate of hydrolysis of chloroacetates and bromoacetates by plasma and erythrocyte cholinesterase. Incidentally to the determination of the affinity ratios we have measured the limiting rates of hydrolysis of the haloesters by the two cholinesterases. The results are presented in Table II, which gives the averaged results of several experiments. The rate of hydrolysis is expressed as a percentage of the rate of hydrolysis of a suitable standard substrate; acetyl choline in the case of the plasma enzyme, acetyl-β-methyl choline in the case of the erythrocyte enzyme. A comparison with our previously published data shows that these haloesters are hydrolysed remarkably rapidly in comparison with other aliphatic esters and approach or exceed the rate of hydrolysis of choline esters. We have not attempted to give a theoretical explanation of these facts.

TABLE II

RATE OF HYDROLYSIS OF HALOACETATE ESTERS

Ester	Plasma enzyme		Erythrocyte enzyme	
	Rate of hydrolysis (% of ACh rate)	No. of times faster than corr. propionate	Rate of hydrolysis (% of MCh rate)	No. of times faster than corr. propionate
		Chloroacetates		
n-butyl	206	13.0	60	3.8
iso-amyl	210	5.7	104	3.6
		Bromoacetates		
n-butyl	263	16.5	87	5.5
iso-amyl	270	7.3	100	3.5

ACh = acetyl choline
MCh = acetyl-β-methyl choline

DISCUSSION

Summarizing, in the first paper of this series[3], the essential differences between the cholinesterases of human blood, we emphasized that while the occurrence of a choline-like configuration in the substrate is necessary for rapid hydrolysis by both enzymes, they differ in their response both to changes in acyl group size and to chain branching in the C–1 and C–2 positions. Such differences suggest that the configuration of the

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active centres in the two enzymes is by no means identical, but do not provide a basis for a simple model which can also account for other differences, such as the occurrence of marked substrate inhibition of the erythrocyte enzyme, but not of the plasma enzyme, by acetyl choline, or the differential inhibition of the two enzymes by DDM and DFP and certain other inhibitors[32, 33]. In this paper we have carried our study of the active centres of these cholinesterases a step further, by showing that the difference in the affinity of choline for the two enzymes and the closely similar affinities of the charged acetyl choline ion and its uncharged C-analogue for the plasma enzyme, is consistent with the existence of a negatively charged atom (*e.g.*, oxygen) in the active centres of the erythrocyte cholinesterase, which is absent from those of the plasma enzyme.

It has been pointed out by AUGUSTINSSON[34] that the fact that the affinity of acetyl choline for cholinesterases of the erythrocyte-type is decreased by the addition of positively charged ions such as clupein, while the negatively charged gum arabic has the reverse effect, suggests that the affinity of the positively charged acetyl choline ion may well be determined by the total negative charge in the enzyme. No such effects in the affinity of acetyl choline for the plasma enzyme have been recorded. These facts are intelligible in the light of the results presented here, for the effective potential exerted by a negative charge associated with the active centres of the erythrocyte enzyme may well be profoundly influenced by changes in the ionic composition of the medium whereas no such change would be expected of active centres, such as those of the plasma enzyme, in which a negatively changed group is apparently absent.

This hypothesis is supported by our finding that the increase in affinity which results when the methyl group of a propionate ester is replaced by halogen is greater for the erythrocyte enzyme than for the plasma enzyme. This difference in behaviour clearly suggests that there is an additional component in the interaction energy of the erythrocyte enzyme and its substrate, though calculations based on the assumption that this was due to ion-dipole interaction did not lead to satisfactory quantitative agreement with experiment.

In the case of the plasma enzyme, where enzyme substrate interaction is likely to be of a kind uncomplicated by ionic effects, calculations based on the assumption that interaction was chiefly due to dispersion forces have had some success. It is realized however that the number of compounds studied is small and that a longer series will have to be investigated before conclusions can be other than tentative.

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SUMMARY

1. It is pointed out that while the absolute value of the energy of attraction of an enzyme for an inhibitor or substrate molecule cannot be evaluated, differences between the energies of attraction of an enzyme and two closely related substrates, or between two closely similar enzymes and an inhibitor may be. An expression is given relating this difference in the energy of attraction to the ratio of the affinity constants.

2. Several applications of this method to the cholinesterases of human blood are described.

3. It is shown that the observed ratios of (a) the affinities of choline for the erythrocyte and plasma enzymes, and (b) of the affinities of the positively charged acetyl choline ion and its uncharged carbon analogue for the plasma enzyme are consistent with the notion that there exists in the active centres of the erythrocyte enzyme a negatively charged atom (*e.g.*, oxygen) which is absent from the active centres of the plasma enzyme.

4. An attempt has been made to study the nature of the forces operating between these cholinesterases and certain aliphatic substrates by studying the effect on the affinity constant of replacing the methyl group of propionate by chlorine or bromine, thus changing the electrical properties of the acyl group without significantly altering its size. In the case of the plasma enzyme, where enzyme-substrate interaction is likely to be uncomplicated by the existence of a negatively charged group in the active centre, fair agreement with experiment was obtained when the value of the haloacetate/propionate ratio was calculated on the basis of the assumption that the interaction was chiefly due to dispersion forces. With the erythrocyte enzyme, the agreement was less satisfactory even when allowance was made for the effect of ionic interaction.

RÉSUMÉ

1. La valeur absolue de l'énergie d'attraction d'un enzyme pour un inhibiteur ou pour un substrat ne peut pas être évaluée. Par contre, on peut évaluer la différence entre les énergies d'attraction d'un enzyme pour deux substrats très voisins ou entre un inhibiteur et deux enzymes similaires. Ces différences entre les énergies d'attraction peuvent être exprimées en fonction des constantes d'affinité.

2. Nous avons décrit plusieurs applications de cette méthode à l'étude des cholinestérases du sang humain.

3. Les valeurs expérimentales, (a) du rapport — affinité de la choline pour la cholinestérase des érythrocytes/affinité pour la cholinestérase du plasma —, (b) du rapport — affinité de l'ion acétylcholine, de caractère électropositif, pour la cholinestérase du plasma/affinité de son analogue carbonique non chargé pour la même cholinestérase —, sont compatibles avec l'idée que le centre actif de l'enzyme des globules rouges contient un atome chargé négativement (p. ex.: oxygène) que le centre actif de l'enzyme plasmatique ne contient pas.

4. Nous avons essayé d'étudier la nature des forces agissant entre ces cholinestérases et certains substrats aliphatiques en suivant les variations de la constante d'affinité au cours du remplacement du radical méthyle du propionate par le chlore ou le brome, remplacement qui s'accompagne d'une modification des propriétés électriques du radical acyle sans modification notable de ses dimensions. Dans le cas de l'enzyme plasmatique, où l'interaction enzyme-substrat n'est vraisemblablement pas compliquée par l'existence d'un radical électronégatif au niveau du centre actif, nous avons calculé le rapport — constante d'affinité des dérivés halogénés de l'acétate — constante d'affinité du propionate — en admettant que l'interaction est essentiellement due aux forces de dispersion: les valeurs obtenues et les valeurs expérimentales concordent de manière satisfaisante. Dans le cas de l'enzyme des globules rouges, la concordance est moins bonne, même si l'on tient compte des interactions ioniques.

ZUSAMMENFASSUNG

1. Der absolute Wert der Attraktionsenergie eines Fermentes für ein Inhibitor- oder ein Substratmolekül ist nicht bestimmbar; messbar sind jedoch Unterschiede in den Attraktionsenergien: (a) eines Fermentes für zwei chemisch nahe verwandte Substrate und (b) zweier nahe verwandter Fermente für ein und denselben Hemmstoff. Ein Ausdruck wird abgeleitet für die Beziehung zwischen solchen Unterschieden in der Attraktionsenergie und dem Verhältnis der Affinitätskonstanten.

2. Anwendungen dieser Methode auf die Untersuchung der Cholinesterasen des menschlichen Blutes werden beschrieben.

3. Die folgenden Grössen wurden bestimmt: (a) das Verhältnis der Affinität des Cholins für die Erythrozytencholinesterase zu dem für die Plasmacholinesterase und (b) das Verhältnis der Affinität des positivgeladenen Acetylcholin-Ions zu dem des entsprechenden nichtgeladenen C-Analogs. Die gefundenen Werte sind vereinbar mit der Interpretation, dass das "aktive Zentrum" des

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Erythrozytenfermentes ein negativ geladenes Atom (z.B. ein Sauerstoffatom) enthält, welches im "aktiven Zentrum" des Plasmafermentes abwesend ist.

4. Um die Art der Kräfte zu untersuchen, die zwischen diesen Cholinesterasen und gewissen aliphatischen Substraten wirksam sind, haben wir die Änderung in der Affinitätskonstante untersucht, wenn man die Methylgruppe im Propionat durch Chlor oder Brom ersetzt. Eine solche Substitution ändert die elektrischen Eigenschaften der Acylgruppe, ohne ihre Grösse merklich zu beeinflussen. Für das Plasmaferment, wo die Beziehung Ferment-Substrat vermutlich nicht durch die Existenz einer negativ geladenen Gruppe im aktiven Zentrum des Fermentes kompliziert ist, ergibt sich eine befriedigende Übereinstimmung mit dem Experiment, wenn man in der Berechnung des Verhältnisses Halogenacetat/Propionat annimmt, dass die Wechselwirkung zwischen Ferment- und Substratmolekülen sich in der Hauptsache durch Dispersionskräfte erklärt. Für das Erythrozytenferment war die Übereinstimmung weniger befriedigend, selbst wenn man eine Ionenkomponente der Wechselwirkung in Rechnung setzt.

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