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To Dr. Bernard Belleau I express my esteem for continuous guidance and patient support during my graduate years. He personally taught me the challenge of research and its rewards in personal satisfaction.

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ABSTRACT

The results of this thesis may be divided into three parts. First, an attempt was made to gain some insight into the stereochemical properties of the Michaelis complex between Acetylcholine (ACh) and Acetylcholinesterase (AChE) through the application of secondary deuterium isotope effects. Four deuterated acetylcholine isomers were synthesized and their Michaelis constants determined. The possibility of the operation of deuterium isotope effects on the binding of certain inhibitors was also explored. It was verified that AChE cannot catalyze the hydrolysis of acetal linkages.

Second, a detailed study of the nature and magnitude of the interaction of the acetate methyl group of acetylcholine with AChE was undertaken. This was made possible by the availability in our laboratories of a wide variety of quaternary salts of the 1-3, dioxolane series. Changes in the geometry and the absolute configuration of the various substituents on the ring of these inhibitors led to some interesting conclusions with regard to the stereospecificity of the enzyme. The theoretical studies of Lionel Salem on London dispersion forces was of value in quantitatively evaluating the magnitude of the forces exerted by the enzyme on the ester methyl group of the substrate.

Finally, the work of Cohn and Edsall on the quantitative estimation of hydrophobic interactions served as a basis for the interpretation of structure activity relationship amongst AChE inhibitors.

INTRODUCTION

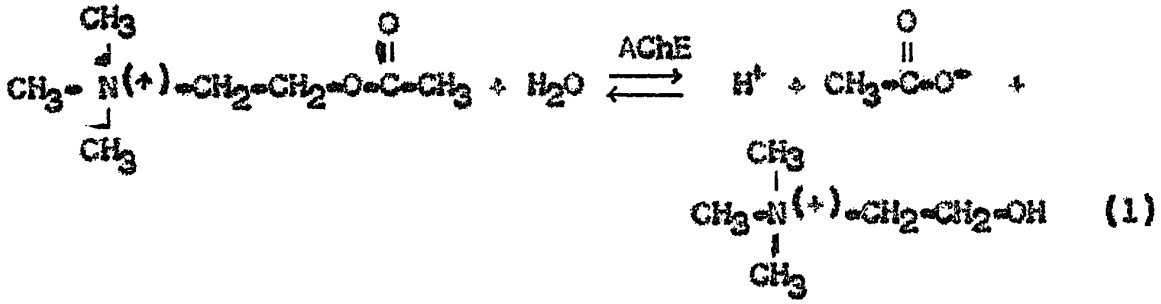
A. REVIEW ON ACETYLCHOLINESTERASE

Introduction

We shall first review the available data on the structure of the active sites of acetylcholinesterase (AChE) and its mode of action with substrates and inhibitors. The term active sites refers to those regions of the enzyme surface where the substrate is localized and activated during enzymatic action. No attempt will be made to include information on the enzyme per se, or its substrates and inhibitors which does not directly contribute to an understanding of the structure of the active site or its reactivity.

The Interaction of AChE with Substrates

AChE, the predominant acetylcholine (ACh) decomposing enzyme of nervous tissue and erythrocytes, is distinguished from other esterases by a number of characteristics. The most essential is its substrate specificity. Its natural substrate is ACh, and this ester is split according to equation (1).



(1)

Propionylcholine is split at a lower rate, and

butyrylcholine is hardly split at all; in fact it strongly inhibits the enzyme.[1]

AChE has been isolated for purposes of biological investigations mainly from the electric organs of certain marine organisms and from erythrocytes. The isolation methods have been recently described in a review by Augustinsson[2] which should be extended to include the procedure of Claire Lawler.[3] In the same review methods for the estimation of the enzymatic activity have been summarized.

It was generally agreed that the interaction of AChE with the substrate can be adequately described by the familiar formulation proposed by Michaelis-Menten

$$EH + S \underset{k_1}{\overset{k_2}{\leftrightarrows}} EHS \longrightarrow EH + \text{products} \qquad (2)$$

where EH is the free enzyme and EHS is the enzyme-substrate addition complex. It is now recognized that during the hydrolysis of ACh, an acetylated enzyme is formed from EHS leading to the liberation of choline. The acetylated enzyme is subsequently split by water to give acetic acid and free enzyme. The remarkable efficiency of the enzymatic attack of ACh is reflected in the very high turnover number of the enzyme. The T.N. is defined as the number of substrate (ACh) molecules reacting per minute per active site.

In order to find the T.N. of eel AChE, Michel and Krop[4] estimated the number of active sites per mg. of AChE by inhibiting it with ^{32}P labelled diisopropyl phosphoro-

fluoridate ($DF^{32}P$). Assuming that every active site reacts irreversibly with one molecule of D.F.P., the amount of ^{32}P per mg. of completely inhibited enzyme preparation will give the number of active sites per mg. They calculated a T.N. of 4.9×10^5 molecules ACh/min/active site (pH 7.4, temp. 38°C, ionic strength 0.14). It should be noted that the optimum pH for the enzyme is 8.4.

It is also generally accepted that two functional groups at the active site of the enzyme are essential in bringing about the hydrolysis of the substrate; the esteratic site where the ester bond is activated, and the anionic site, consisting of one or more negative groups which interact through ionic bonds with the cationic nitrogen of the choline residue. It is clear also that Van der Waals forces are operative.

In the following sections we shall deal consecutively with the main factors which determine the enzyme-substrate interaction.

These factors can be summarized as follows and will be discussed separately:

a. The concentration of the substrate;

b. The structure of the substrate;

c. The effect of pH;

d. The effect of the presence of inorganic ions;

e. The effect of the temperature.

a. The Concentration of the Substrate

I. Substrate Inhibition

When the initial reaction velocity (V) for the hydrolysis of ACh by AChE is plotted against the concentration of the substrate (S) a curve is obtained (Fig.1, p.5) which deviates from the typical rectangular hyperbola of enzyme reactions obeying Michaelis-Menten kinetics (Fig.2, p.5).

It can be seen in Fig.1 that starting at low substrate concentrations, the initial reaction velocity increases until an optimal concentration of substrate is reached. It has been found that this concentration lies at approximately 3×10^{-3}M ACh. At higher concentrations the reaction is inhibited to produce a "bell-shaped" curve.

The phenomenon was first noticed by Alles and Hawes[5] for red cell AChE and was explained by assuming that an inactive complex was formed consisting of one mole of enzyme and two or more moles of substrate. The formation of this complex by ACh at high substrate concentrations is readily explained by assuming that at high substrate concentrations each of two sites on the enzyme active surface attracts a substrate molecule. It is understandable that in this complex the substrate would not be in a favorable steric position for activation of the ester bond, resulting in a decrease of enzymic activity. More recently, however, Krupka and Laidler[6] have shown that substrate inhibition follows a different mechanism. Their results show that substrate

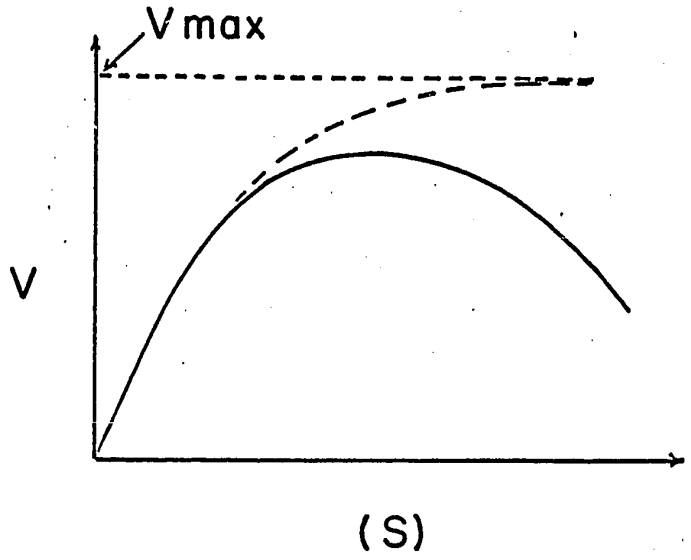


Fig. 1 A schematic substrate concentration curve showing inhibition by substrate.

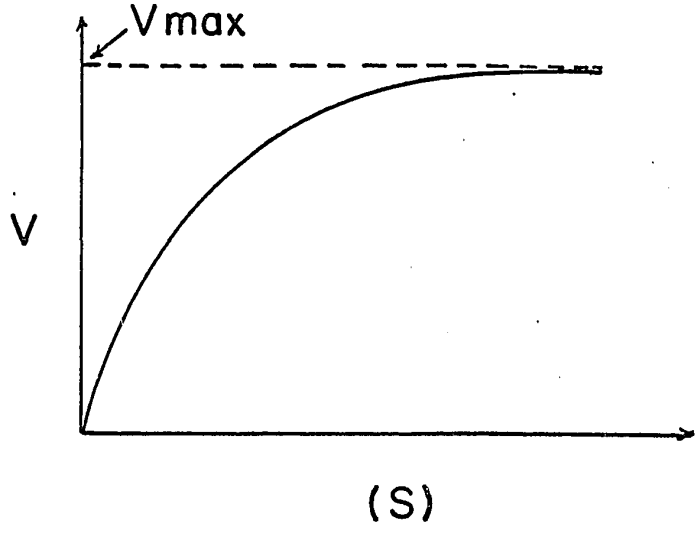


Fig. 2 Hyperbolic form of typical substrate concentration curve.

inhibition is due not to the formation of a complex in which two substrate molecules are attached to the enzyme, but to the attachment of a substrate molecule to the acetyl enzyme with blocking of the deacetylation process.

It can be shown from the steady-state equations that if an inhibitor blocks deacetylation, the square of the concentration of substrate, [S] opt., corresponding to the maximum velocity, should vary linearly with [I]; if it does not, [S] opt. itself should vary linearly with [I].

II. Kinetics of enzyme-substrate interaction

It has been mentioned that the simplest way of representing the action of AChE according to the classical Michaelis-Menten theory (Equation 2) is unsatisfactory because it does not account for the formation of an acetyl enzyme intermediate.

$$E.H.S. \xrightarrow{k_4} E\text{-acyl} + \text{alcohol} \xrightarrow[H_2O]{k_5} EH + \text{acid} + \text{alcohol} \quad (3)$$

Equation 3 describes the phenomenon in a more satisfactory manner. This is not in contradiction with the formulation of equation 2, but it implies that the k_3 term in the equation is complicated because it is composed of at least two reaction velocity constants (k_4 and k_5).

According to (2), the reaction is first order with respect to enzyme concentration and the velocity $v = k_3(EHS)$. It is also first order with regard to substrate at low

substrate concentration as expected from (2) and accounts for the left hand part of the curve in Fig.2. At higher substrate concentrations it should become zero order, as it does in Fig.2, but in fact it follows the pattern shown in Fig.1, and this is due to inhibition by high concentrations of substrate. The substrate concentration curve is bell-shaped but the curve has a flat maximum that corresponds to a fairly wide range of substrate concentrations. Therefore, also in this case, zero order kinetics prevail over a considerable range. However, the maximum activity can no longer be represented by $V_{max} = k_3(EH_t)$ as it is in classical Michaelis-Menten kinetics, where (EH_t) represents the concentration of the initial enzyme present and V_{max} the maximal velocity.

The substrate inhibition of AChE can be represented as follows, assuming the possibility of the formation, in addition to the normal Michaelis complex E.H.S., of an inactive complex EHS_2 (super complex) consisting of one enzyme and two substrate molecules.

$$E.H.S. + S \rightleftharpoons E.H.S_2 \text{ (diss. constant } K_2) \qquad (4)$$

$$(EHS_2) = \frac{(EHS)\,(S)}{K_2}$$

The reaction velocity under conditions of substrate inhibition is given by equation 5.

$$v = \frac{V_{max}\,(S)}{K_m + (S) + \frac{(S)^2}{K_2}} \qquad (5)$$

For the steady state $\frac{d(E_t H_t S_t)}{dt} = 0$ of equation (2) where the rate of formation and removal of EHS are equal

$$(EH)(S)\, k_1 = k_2\,(EHS) + k_3(EHS) \qquad (6)$$

For the concentration of free enzyme (EH)

$$(EH) = (EH_t) - (EHS) - \frac{(EHS)(S)}{K_2}$$

Assuming that $(EH_t) \ll (S)$, the expression (S) in the formula is not corrected for enzyme-bound substrate and is taken to be equal to the total substrate concentration. Substitution of (EH) in (6) and rearrangement gives

$$\frac{(EHS)}{(EH_t)} = \frac{(S)}{\frac{k_2 + k_3}{k_1} + (S) + \frac{(S)^2}{K_2}}$$

substitution of $\frac{(EHS)}{(EH_t)} = \frac{v}{V_{max}}$ and $\frac{k_2 + k_3}{k_1} = K_m$ (Michaelis-Menten constant) yields

$$v = \frac{V_{max}\,(S)}{K_m + (S) + \frac{(S)^2}{K_2}} \qquad (7)$$

Formula (7) describes the bell-shaped curve as represented in Fig.1. It is obtained when (S) or log (S) is plotted against v. In the latter case the "bell" is symmetrical around an axis corresponding to the optimal substrate concentration. Formula (7) reduces to (8) when K_2 is very large or (S) very small.

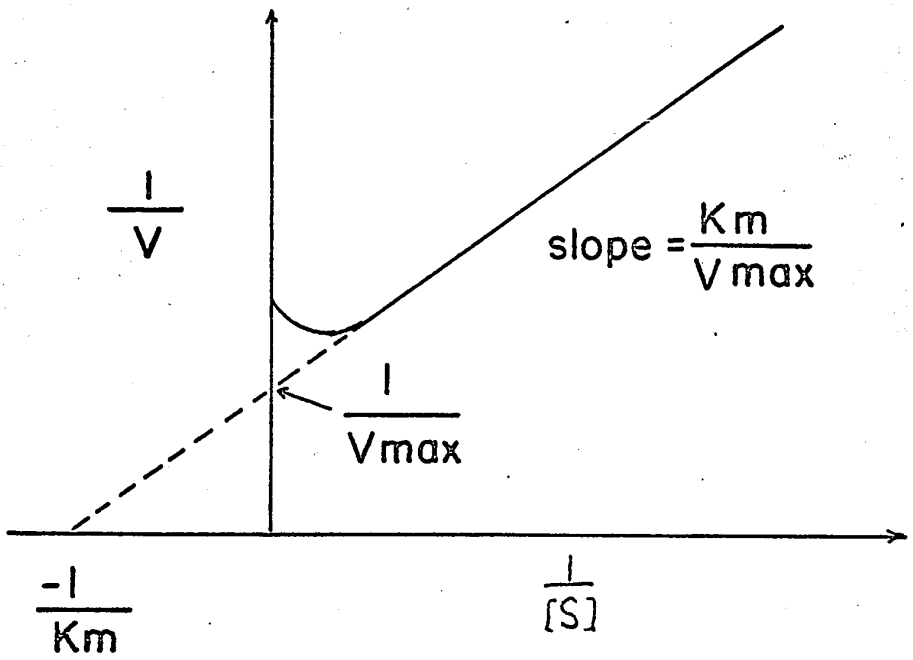


Fig. 3 Determination of K_m and V_{max} in the case of substrate inhibition.

$$v = \frac{V_{max}\,(S)}{K_m + (S)} \qquad (8)$$

This is the familiar Michaelis expression for a rectangular hyperbola; here K_m = [S] at half maximal velocity. Using this expression, K_m and V_{max} may be easily computed, e.g., from a Lineweaver-Burk plot of 1/v against 1/(S) (Fig.3, p.9); for this purpose, the linear segment of the curve in Fig.3 corresponding to low values of [S] should be used. Because of substrate inhibition, the maximal velocity observed experimentally never actually reaches V_{max} as may be seen from the same figure.

The K_m which is read from the graph is equal to $\frac{k_2 + k_3}{k_1}$ from equation (2). It is not equal to k_2/k_1, i.e. the dissociation constant of the Michaelis complex. It would be of considerable interest to know this dissociation constant because it could provide valuable information on the nature of the ACh-AChE Michaelis complex. Fortunately the K_m computed in this simple way can serve a useful purpose since it has been shown that for many enzyme-substrate systems $K_m = \frac{k_2 + k_3}{k_1}$ simplifies into $K_m = k_2/k_1$, because frequently $k_2 \gg k_3$. (Laidler[7]).

b. The Structure of the Substrate

1. Substrate Specificity for AChE

By studying various modifications of the substrate, requirements for the formation of the enzyme substrate-complex may be ascertained to some degree.

In a homologous series of choline esters, ACh is the best substrate, followed closely by propionyl choline. Augustinsson[1] reported on the pS-activity relationship for the choline esters, including acetyl-β-methyl choline. Whittaker[8] studied the relation between the length of the acetyl chain and the speed of hydrolysis. It is interesting that no inhibition by high substrate concentrations was observed for dimethylaminoethyl acetate as shown by Wilson[9]; the pS activity curve of these substrates does not have a "bell shape". Bergmann and Shimoni[10] reported that the same applies to non-charged aliphatic esters amongst which is the carbon analogue of ACh, β,β-dimethylbutyl acetate[11]. This ester is notable for its rapid hydrolysis by AChE, showing that binding with an anionic site is not an absolute requirement for proper binding of the substrate at the active site. In the case of these compounds the absence of substrate inhibition is readily explained on account of the lack of a cationic entity in the molecule; a charged group is necessary for the formation of an inactive addition complex (EHS_2).

Carboxyl acid anhydrides[9] are hydrolysed by AChE and thus have a bearing on the concept of an acyl enzyme intermediate during catalysis. Substituted phenyl esters are also hydrolysed[12]. The interesting hydrolysis of thiol-acetic acid to hydrogen sulphide and acetic acid should be noted[13].

II. Stereochemical Specificity

Augustinsson and Isacsohen[14] have shown that only the D-form of acetyl-β-methyl choline is attacked by AChE. According to Amman and Meyer[15], AChE from brain tissue of various species hydrolyses preferentially the L-configuration of the choline ester of mandelic acid (possessing an asymmetrical C-atom in the acid residue). Bergmann et al[16] found that for the inhibition of AChE by amino acids the L-configuration was required. Surprisingly little work has been done on the stereospecificity of the enzyme.

III. Interpretation of the Substrate Specificity of AChE with respect to the Properties of the Active Sites

Wilson[9] found very good agreement between the difference in dissociation constants of AChE with ethyl acetate, isoamyl acetate, and ACh and the expected free energy differences obtainable from estimates of the Van der Waals contributions to the forces of attraction exerted by the methyl groups and the coulombic interactions between the positive nitrogen and the anionic site of the enzyme. These studies also showed that the substrates; acetic anhydride and dithiolacetic acid (which lack a cationic entity) have a K_m which is of the same order as that of ACh. This result underscores the importance of the electrophilic carbon in the interaction of substrates with the esteratic site. Moreover, Wilson[9] demonstrated that the introduction of a fourth methyl group into the trimethylammonium ion did not influence the binding constant with AChE. On the other hand,

the difference in K_m between dimethylaminoethyl acetate and ACh suggests that the interactions are of a complicated nature. This, coupled with the fact that dimethylaminoethyl acetate does not lead to substrate inhibition, prompted Wilson to conclude tentatively that when substrate inhibition occurs, deformation of the AChE molecule would take place. Friess and Balbridge[17] studied the substrate properties of the cis-isomer of 2-trimethyl-amino-cyclopentanol acetate and its corresponding six-membered analogue. They found that these esters are better substrates than ACh and concluded that the distance between the anionic and the esteratic site on the AChE surface amounts to approximately 2.8°A, whereas the mean separation distance between the N and the O-atom in the $N-CH_2-CH_2O$-chain of ACh was calculated to be 2.3°A in the bound molecule. In this connection it is interesting to recall the conclusion of Bernhard[18] that ACh appears to be 1.0°A too short; this conclusion was based on electrostatic energy calculations on the assumption (unjustified, however) that the excellent inhibitor physostigmine provides a veracious reflection of the enzyme surface. Whittaker[19] pointed out that if an inhibitor like neostigmine with a longer carbonyl-nitrogen distance and an affinity for AChE 10^4 times greater than ACh was taken as assuming an unstrained configuration the calculated difference in binding energies would reflect an appreciable degree of deformation in the bound substrate.

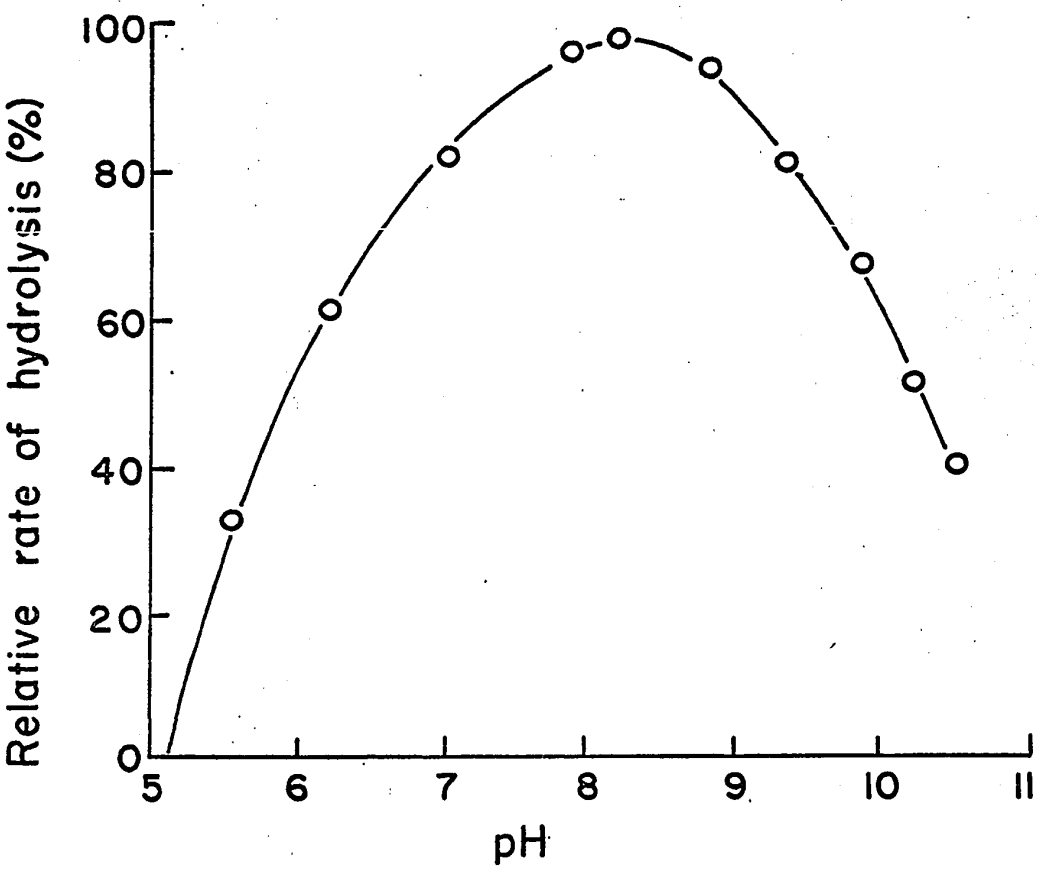


Fig. 4 Hydrolysis of ACh by AChE as a function of pH. Substrate ACh 5 x 10^{-3}M.

c. The Effect of the pH

During the catalytic action of AChE the pH of the medium can affect the enzyme, the substrate or both. As far as the substrates are concerned, the main role of the pH will be to influence the degree of dissociation of the ionizing groups, especially the nitrogen atom of the tertiary amines. When choline is involved, the nitrogen is present in the quaternary form and therefore the amount of charge is essentially pH-independent. Thus when ACh is the substrate,[20] the variations in pH ought to effect changes only in the enzyme (Fig.4, p.14).

Wilson[21] suggested that the bell-shaped curve could be due to the influence of the ionization state of a basic and an acidic group in AChE; the ionization constants were estimated to be pK_a 10.3 and pK_b 6.3 respectively.

The value of 6.3 for the pK_b is generally taken as evidence for the participation of an imidazole residue in the catalytic process. The β-carboxylic group of aspartic acid or the γ-carboxylic group of glutamic acid may be involved in supplying a functional group at the anionic site. It should be noted, however, that the pK's of these groups (3.65 and 4.25, respectively) differ considerably from 10.3. In this connection Bergmann et al[22] have ventured the opinion that protonated imidazole at the esteratic site may influence the dissociation of the

carboxylic group at the anionic site. In connection with their hypothesis that two negative sites may be present on the surface, Bergmann et al have further suggested that the anionic site may contain an -glutamyl- -glutamyl couplet situated near the esteratic site. The problem of the chemical nature of the anionic site remains unsolved.

d. The Effect of Inorganic Ions

Most studies have been concerned with the activation of AChE by divalent cations. Unfortunately, only very few experiments have been performed on purified preparations even though this is necessary to obtain unequivocal results. Nachmansohn[23] showed activation of eel AChE by Ba^{++}, Ca^{++}, Mg^{++} and Mn^{++}, the efficiency increasing in this order.

The effect of storage on the Mg^{++} activation of AChE

pH	Added Mg^{++}, M	Versene M	Activity after dilution		
			0 hr.	21 hr.	60 hr.
7.4	0.016		100	100	100*
7.4	0		15	50	76
8.2	0	0.014	18	79	91

Diluted solution stored at 3-4°. The tests were run at 25.12 ± 0.02° with a substrate concentration of (3.34×10^{-3}M) and phosphate buffer (1.2×10^{-2}M).

*The absolute activity in this standard run with Mg^{++} present was found to have dropped only 1% from that observed at 0 hours.

The above data summarizes the results of Friess et al[24]. A curious effect is shown in the activity of AChE after dilution. The rise in activity in the absence of added metal ions, coupled with the activating effect of versene, suggests that the original preparation contained an inhibitory metal, which gradually dissociated away after dilution. According to Dixon and Webb[25] this probably means that the effect of Mg^{++} in raising the activity to a constant value is due to a displacement of the inhibitor and not due to direct activation.

The activation by univalent inorganic cations (Na^+, K^+ and Li^+) is probably non-specific and due only to an increase in ionic strength. Compared with divalent ions, higher concentrations are usually required to obtain equivalent activation. Moreover, the effect of the divalent ions is additive with respect to that of the monovalent ones. Summarizing the available evidence, it appears that AChE is not a metallo-enzyme and that the activation by Na^+, K^+, Li^+, Mg^{++} and Ca^{++} ions observed in the presence of the usual substrate concentration is highly dependent on the experimental conditions.

e. The Effect of Temperature

Wilson and Cabib[26] applying equation (8) determined the K_m of eel AChE for various substrates and found that it varied little over the temperature range 15° to 30°.

Investigation of the dependence of k_3 on temperature was also very instructive. It had been shown earlier (equation (3)) that the complete mechanism of action for AChE (disregarding inhibition by high substrate concentration) may be written as follows:

$$EH + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EHS \xrightarrow[k_4]{} E\text{-acyl} + \text{alcohol} \xrightarrow[k_5]{H_2O} EH + \text{acid} \qquad (9)$$

$$\xrightarrow[k_3]{}$$

According to this equation k_3, which was defined earlier by the equation $v = k_3(EHS)$, includes at least two reactions with velocity constants k_4 and k_5. Wilson and Cabib[26] tried to establish which part of the overall velocity constant k_3 could be ascribed to k_4 and to k_5, at various temperatures. Using data from the variation of v against [S], plots of log k_3 versus 1/t were obtained. The slope of the curve which is a straight line allows an estimate of the activation energy E. They thus obtained a curve with a downward slope for ACh, whereas for other acetylated substrates of lower affinities (larger K_m) straight lines were observed (Fig.5, p.19). Therefore in the latter case it is likely that the energy of activation is determined by one velocity constant which may be either k_4 or k_5 at all temperatures. All the acetylated substrates used must give an identical acetyl enzyme intermediate and must have correspondingly an identical k_5. The difference in the

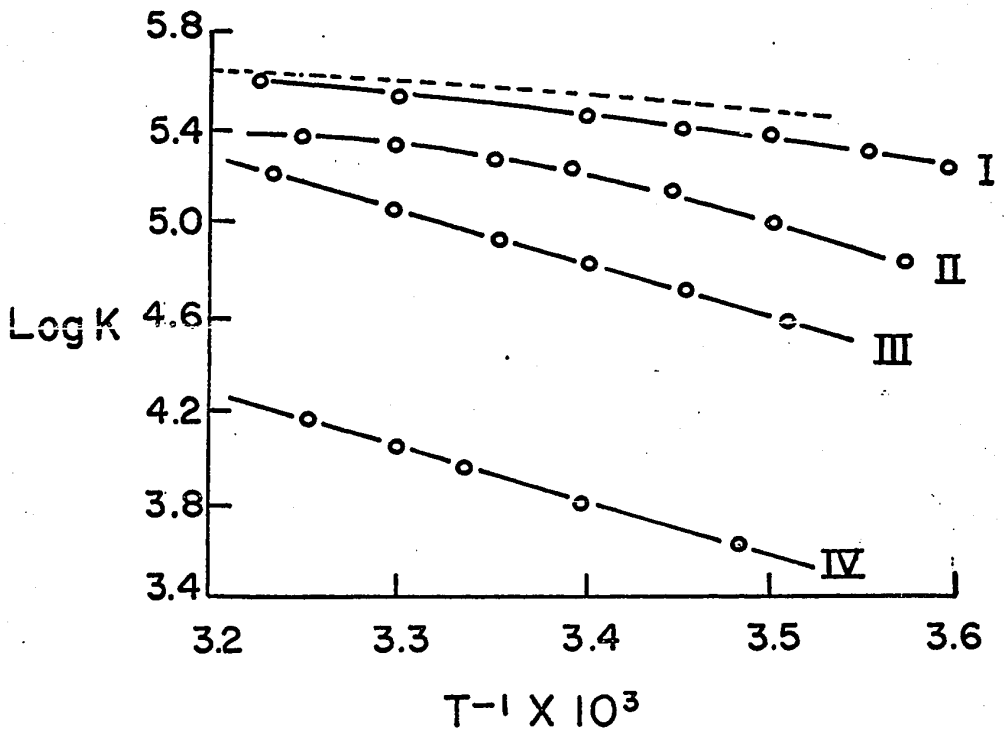


Fig. 5 Arrhenius plot of log K versus T^{-1} for the acetylcholinesterase-catalysed reactions of various substrates. The dotted line is drawn as an estimated asymptote to the curve for substrate I and represents log K_5. Substrates used:

(I) Acetylcholine
(II) Dimethylaminoethylacetate
(III) Methylaminoethylacetate
(IV) Aminoethylacetate

from Wilson and Cabib

slopes observed with different acetylated substrates must therefore be due to variations in k_4, the rate constant for the formation of the acetyl enzyme complex.

The non-linear curve for ACh is likely to be due to varying contributions of k_4 and k_5 depending on temperature. Cohen[27] concluded that for sluggish substrates (high K_m) k_4 is rate limiting; whereas for good substrate (small numerical values of K_m) like ACh, k_5 is rate limiting at high temperature, and k_4 rate limiting at low temperatures (Fig.5, p.19).

The Interaction of AChE with Inhibitors

Inhibitors of AChE may be divided into reversible and irreversible inhibitors. We shall use the term reversible to indicate that the enzyme-inhibitor complex dissociates freely upon the application of dialysis or otherwise, thus showing that there exists an equilibrium between free inhibitor and enzyme. With irreversible inhibitors, no such equilibrium can be established and the enzyme activity does not (at least not readily) return on mere dialysis. The distinction between reversible and irreversible inhibitors is by no means absolute. There can be a continuous transition from reversible inhibitors to irreversible inhibitors.

a. Reversible Inhibitors

I. Kinetics of the Interaction of AChE With Reversible Inhibitors

The reversible inhibitors may be divided into competitive and non-competitive inhibitors. In the case of a competitive inhibitor, the substrate and the inhibitor simply compete for the enzyme active sites or merely hinder each other's attachment to separate sites. The formation of the enzyme-substrate complex will be hampered, but its hydrolysis will be unaltered. Thus, the maximal velocity (using excess substrate) will be equal to V_{max} both in the inhibited and uninhibited cases but K'_m, the apparent Michaelis constant will increase with respect to K_m.

In the case of non-competitive inhibition it is assumed that separate sites on the AChE molecule accommodate the substrate and the inhibitor and they do not influence each other's interaction with the enzyme molecule. However, the rate of hydrolysis will be decreased, because the enzyme molecules which have combined with inhibitors are not as available for the hydrolysis step. In other words, the maximal velocity in the presence of inhibitor, V'_{max}, will be less than V_{max} for the uninhibited enzyme, but K'_m (i.e., the substrate concentration at half V_{max}) will be equal to K_m.

Competitive Inhibition

Competitive inhibition is the most interesting interaction in the present context; in this case substrate

and inhibitor compete for one and the same active site on the AChE molecule. The following reactions take place:

$$EH + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} EHS \xrightarrow[H_2O]{k_3} EH + \text{products} \qquad (10)$$

with $K_m = \dfrac{k_2 + k_3}{k_1}$

[$EHS + S \rightleftarrows EHS_2$ with dissociation constant K_2, and $EH + I \rightleftarrows EHI$ with dissociation constant K_i and inhibitor I]. The most convenient equations are obtained when v, the velocity for the inhibited enzyme is related to v', the velocity in the presence of inhibitor. We find for v:

$$v = \frac{V_{max}\,(S)}{K_m + (S) + \dfrac{(S)^2}{K_2}} \qquad (11)$$

In the presence of inhibitor the rate of hydrolysis will depend entirely on the fraction E.H.S. of the total available (EH_t) that is formed:

$$\frac{v'}{V_{max}} = \frac{EHS}{(EH)_t} \qquad (12)$$

Now $$(EH_t) = EH + \frac{(EH)(S)}{K_m} + \frac{(EH)(S)^2}{K_m K_2} + \frac{(EH)(I)}{K_i} \qquad (13)$$

After substitution of (13) in (12) yielding

$$\frac{v}{V_{max}} = \frac{(EHS)}{(EH)\left[1 + \dfrac{(S)}{K_m} + \dfrac{(S)^2}{K_m K_2} + \dfrac{(I)}{K_i}\right]}$$

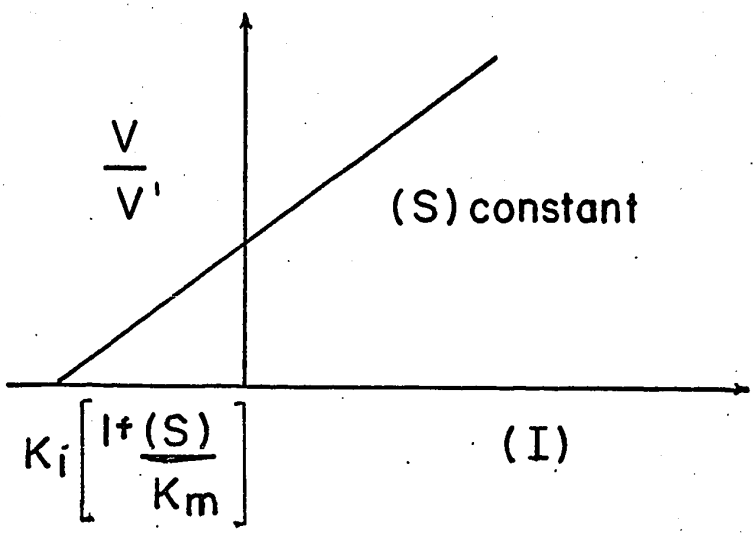


Fig. 6 Schematic plot of V/V' against (I) for competitive inhibition with (S) constant.

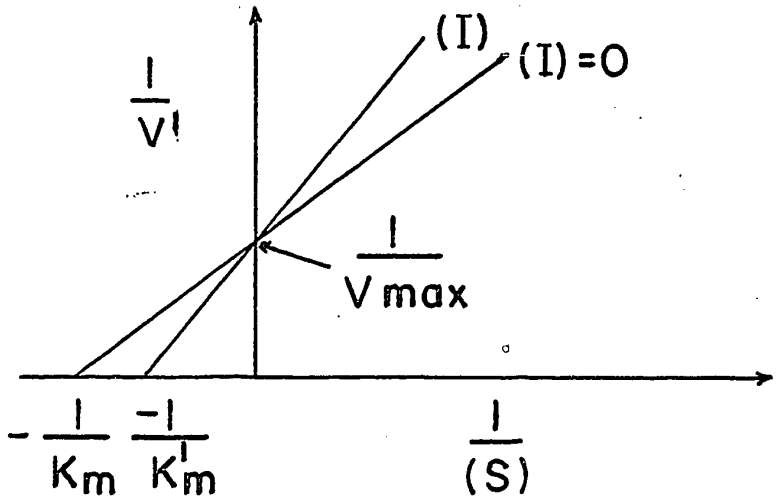


Fig. 7 Schematic plot of 1/V' against 1/(S) for competitive inhibition. The reciprocal of the apparent Michaelis constant $K'_m = K_m\left[1 + \frac{(I)}{K_i}\right]$ is found at the intercept of the curve with the 1/(S) axis and is dependent on (I).

The reciprocal of V_{max} is found at the intercept of the curve with the 1/V' axis and is independent of (I).

and substitution of $\frac{(EHS)}{(EH)}$ by $\frac{(S)}{K_m}$ [reaction (10)] we arrive at

$$v' = \frac{V_{max}\,(S)}{K_m\left[1 + \frac{(S)}{K_m} + \frac{(S)^2}{K_m K_2} + \frac{(I)}{K_i}\right]} \qquad (14)$$ and

$$\frac{v}{v'} = 1 + \frac{(I)}{K_i\left[1 + \frac{(S)}{K_m} + \frac{(S)^2}{K_m K_2}\right]} \qquad (15)$$

When substrate inhibition is neglected,

$$\frac{v}{v'} = 1 + \frac{(I)}{K_i\left[1 + \frac{(S)}{K_m}\right]} \qquad (16)$$

(Fig.6,7, p.23).

With known values for K_m, K_2 and (S), K_i may be calculated. The graph may also be used to read the value for [I] at $v/v' = 2$, i.e., the $(I)_{50}$, or the concentration of inhibitor producing 50% inhibition of the rate. The pI_{50} simply equals $-\log I_{50}$.

Non-competitive Inhibition

Only fully non-competitive inhibition will be treated, where the interactions of the substrate and of the inhibitor with AChE do not mutually influence each other's affinity for the enzyme. It is also assumed that only the EHS complex is active. The enzyme-substrate-inhibitor-complex EHIS and the enzyme-double + substrate-inhibitor-complex $EHIS_2$ are supposed to be as inactive as EHI.

From $EH + I \rightleftarrows EHI$, with dissociation constant K_i, it follows that:

$$\frac{(EH_t) - (EHI)}{(EHI)} = \frac{K_i}{(I)} \quad \text{and}$$

$$(EHI) = \frac{(I)}{K_i + (I)} \cdot (EH_t)$$

Since we are dealing with non-competitive inhibition, all enzyme initially present $(EH)_t$ (including EH and EHI) is also in equilibrium with the substrate. However, only $(EH)_t - (EHI) = \frac{K_i}{K_i + (I)} \cdot (EH_t)$ will be available for the formation of an active, hydrolysable EHS complex. The reaction velocity in the absence of inhibitor is estimated using [equation (11)].

$$v = \frac{V_{max}(S)}{K_m + (S) + \frac{(S)^2}{K_2}}$$

When V'_{max} is the maximal velocity in the presence of inhibitors, it will be related to the V_{max} in the same way that the available enzyme $(EH_t - EHI)$ is related to (EH_t). Thence,

$$V'_{max} = V_{max} \frac{K_i}{K_i + (I)}$$

and

$$v' = \frac{V_{max} \cdot (S)}{K_m + (S) + \frac{(S)2}{K_2}} \cdot \frac{K_i}{K_i + (I)} \qquad (17)$$

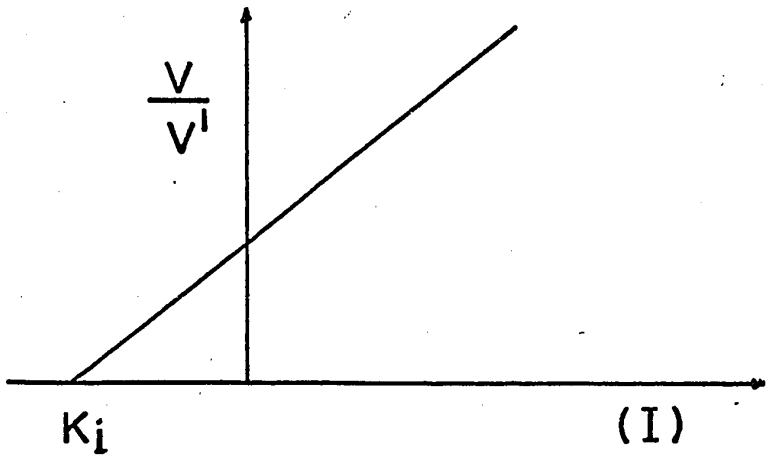


Fig. 8 Schematic plot of V/V' against (I) for non-competitive inhibition.

K_i is found at the intercept of the curve with the (I) axis. The slope of the curve is independent of (S).

The ratio of the reaction velocities is

$$\frac{v}{v'} = \frac{K_I + (I)}{K_I} = 1 + \frac{(I)}{K_I} \qquad (18)$$

It follows from the above equations that for every concentration of inhibitor, v/v' is independent of the substrate concentration. V'_{max} is smaller than V_{max} for the uninhibited enzyme, but K_m is unchanged. Competitive inhibition can be distinguished experimentally from non-competitive inhibition only when the equilibrium substrate-inhibitor-enzyme is established with sufficient speed (Fig.8, p.26).

II. The Structure of Reversible Inhibitors in Relation to the Active Site of AChE

(1) In General:

Reversible, fully competitive inhibitors may react with the anionic site, the esteratic site or both. Representative for the first class of inhibitors of AChE are the alkylated ammonium ions. From the K_I values of mono-, di- and trimethylammonium derivatives, Wilson[9] concluded that the Coulomb interaction is augmented by dispersion forces at a rate of a change in ΔF of 1.2 Kcal. per mole per methyl group; the addition of the third methyl group did not increase the affinity of the N^+ for AChE.

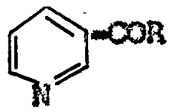
Bergmann and Shimoni[28] showed that there is a linear relationship between the pI_{50} and the number (n) of carbon atoms of a homologous series of monoquaternary

ammonium salts of the general formula:

$$(CH_3)_3\text{-}N^+\text{-}(CH_2)_n\text{-}CH_3$$

A change in ΔF per methylene group of 300 cal/mole was calculated at 20° for AChE.

Bergmann et al[16], comparing the inhibitory properties of amides, dipeptides, esters, ketones and nitriles found that compounds possessing an electrophilic C atom interact with a basic group of the esteratic site and thus cause competitive inhibition. A fair correlation exists between electrophilic character and inhibitory power in the nicotinic acid series where R in the formula



is substituted. The order of increasing electrophilicity of the carbon atom for substituents (R) is: $O^- < NH_2 < OET < NMe_2 < Me$, but the last three are nearly equal; the order for inhibition is: $O^- < NH_2 < NEt_2 < Me < OEt$.

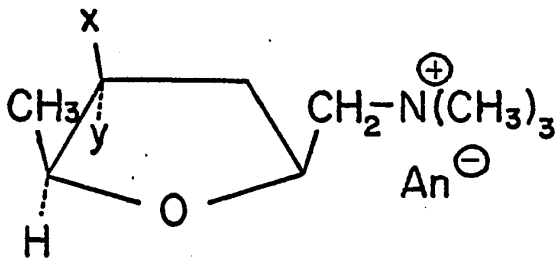
(ii) Stereospecificity towards reversible inhibitors

The Muscarine Series

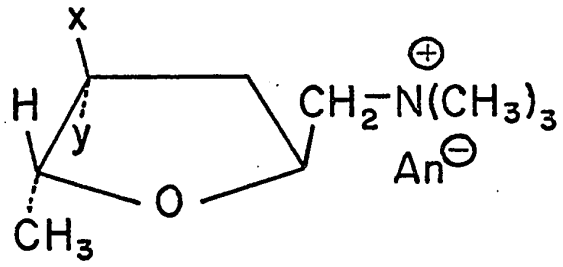
Isomers of the quaternary amine salt muscarine and their acetyl derivatives, have been studied by Friess et al[29]. They are weak to moderate inhibitors of the in vitro acetylcholinesterase-acetylcholine system. Some valuable comparisons of intrinsic inhibitory strengths can be drawn from the data in Table I (p.29,30). Recalling that comparative inhibitory strengths are inversely related to

Table 1

Inhibition of AChE-ACh by Muscarine Congeners



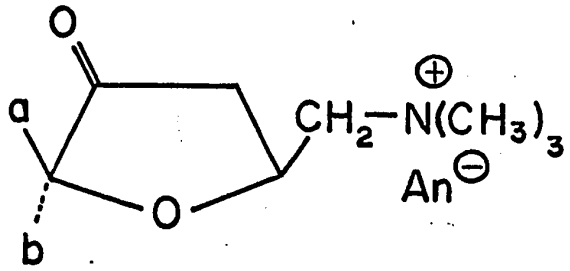
	Competitive Inhibition $K_i \times 10^4$ M/l
(M)x = H, y = OH. DL Muscarine Chloride	6.3
(EM)x = OH, y = H. DL epi Muscarine Chloride	3.0
(AcM)x = H, y = OAc. DL acetyl Muscarine Iodide	2.3
(AcEM)x = OAc, y = H. DL acetyl epi Muscarine Iodide	1.4



(AM)x = OH, y = H. DL allo Muscarine Iodide	7.8
(EAM)x = H, y = OH. DL epi-allo Muscarine Chloride	10.1
(AcAM)x = OAc, y = H. DL acetyl allo Muscarine Iodide	2.7
(AcEAM)x = H, y = OAc. DL acetyl epi-allo Muscarine Iodide	3.9

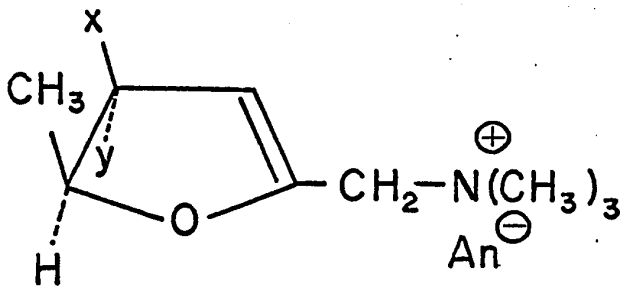
The abbreviations M, EM, AM, and EAM accompanying the structural formulaes refer in sequence to the normal muscarine, epi, allo, and epi-allo configurations of the substituents with respect to the ring, with each compound prepared as the DL modification.

Table 1 (cont'd)

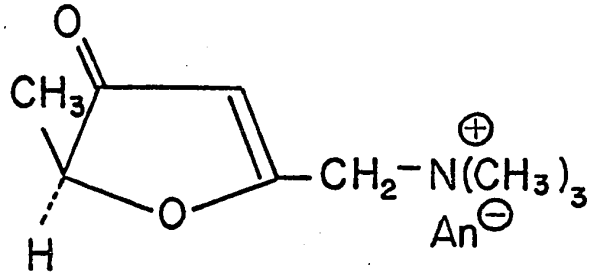


Competitive Inhibition
$K_i \times 10^4$ M/l

a = CH_3, b = H. DL Muscarone Iodide 2.5
a = H, b = CH_3. DL allo-Muscarone Iodide 1.6



x = OH, y = H. DL(cis) 4-5 dehydro Muscarine Iodide 3.4
x = H, y = OH. DL(trans) 4-5 dehydro Muscarine Iodide 1.9



DL 4-5 Muscarone Iodide 10.9

the K_1 values, the sequence in efficiency is as follows: for the OH Series, EM > M > AM > EAM with K_1 values in the relative ratios of 1:2.1:2.6:3.4; for the OAc Series, Ac-EM > Ac-M > Ac-AM > Ac-EAM with K_1 values in the relative ratios 1:1.7:2.0:2.9.

It is seen that in both series the response of AChE to stereochemical configuration of the inhibitor is quite precisely graded, and further that the relative sequences of efficiencies is a function of the orientation of the substituents. A striking parallel exists between the two series.

Further, the effect of acetylation of a given muscarine isomer is seen to cause an abrupt elevation of inhibitory power by an average factor of about 2.6; the K_1 ratios (and accordingly the reciprocal of the strength ratios) in the four OH-OAc pairs, M/Ac-M, EM/Ac-EM, AM/Ac-AM, and EAM/Ac-EAM are, respectively, 2.8, 2.2, 2.9 and 2.6.

An effect of a <u>cis</u> and/or a <u>trans</u> configuration is also established by comparing muscarine with muscarone or 4-5 dehydromuscarine. In both series the <u>cis</u> arrangement provides the best inhibitors.

Oxidizing the 4-5 dehydromuscarine to 4-5 dehydromuscarone results in a significant decrease in inhibitory power[30].

b. Irreversible Inhibitors

Since our research project is concerned mainly with reversible inhibitors, the irreversible inhibitors will be mentioned only briefly.

Many reversible inhibitors have an irreversible character as a result of very slow dissociation of the enzyme-inhibitor complex. In the case of irreversible inhibitors, however, there is no true dissociable enzyme-inhibitor complex at all, but a chemical reaction takes place to form a compound from which the inhibitor is never recovered in unaltered, active form, even though the active enzyme may be set free by reactivation.

The most important group of irreversible anti-ChE agents is formed by the organophosphorus compounds. These compounds act by phosphorylation of enzymes leading to irreversible inhibition. For several enzymes the reaction is stoichiometric and may be described as follows:

$$EH + P - X \longrightarrow E - P + H^+ + X$$

The reaction is accompanied by proton release[31], Michel and Krop[4], using ^{32}P labelled diisopropyl phosphoro-fluoridate ($DF^{32}P$), showed that in the case of AChE the phosphorus atom is tightly bound to the enzyme. The chemical nature of the interaction of enzymes with organophosphorus inhibitors is revealed by the high values (approx. 10 to 15 Kcal/mole) found for the activation energy.

Wilson and Bergmann[32] postulated that phosphorylation of AChE by inhibitors would be the counterpart of

acylation of the enzyme by substrates, the difference being that in the latter case substrate hydrolysis would follow whereas the phosphorylated enzyme would be stable. Both phosphorylation and acylation would be localized at the same esteratic site as shown by the strong protection afforded by substrate against attack by organophosphorous agents. A number of phosphoryl-AChE complexes are subject to spontaneous hydrolysis in aqueous medium. This reaction is accelerated in the presence of nucleophilic agents, called reactivators, such as choline, imidazole, pyridine, and particularly hydroxylamine. The reaction is accompanied by recuperation of the enzymatic activity[33].

Mode of Interaction of ACh and Inhibitors With the Active Site of AChE

I. Mechanism of the Hydrolytic Process

The active surface has two functionally and spatially separated subsites: an "anionic" site and an "esteratic" site. The former attracts the cationic group of the substrate by Coulombic and Van der Waals' forces. The esteratic site has an acidic (Ac) and a basic or nucleophilic group (Ba). The nucleophilic group eventually forms a covalent bond with the electrophilic carbon of the carbonyl group.

The following mechanism has been proposed for the hydrolytic process[16]:

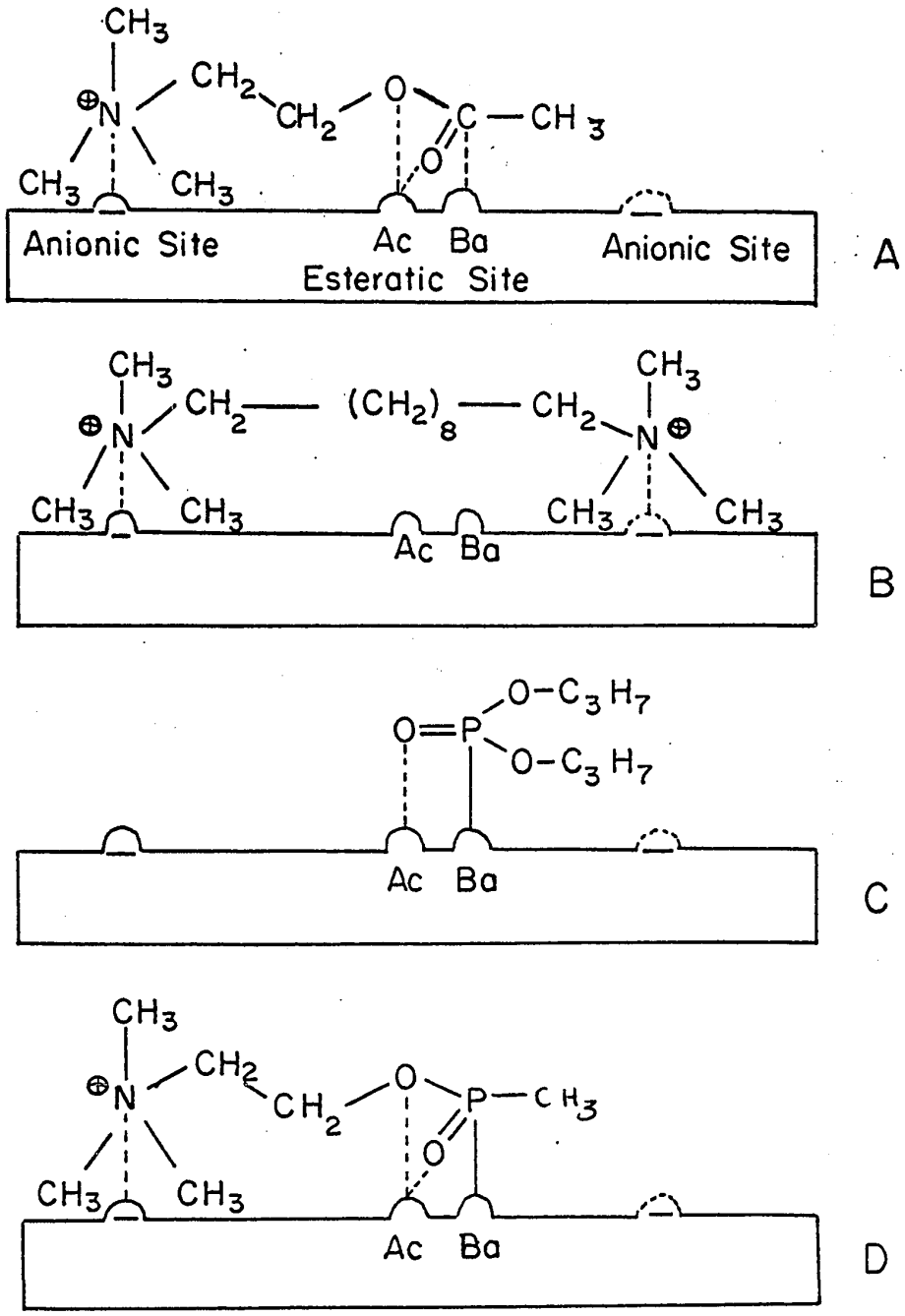
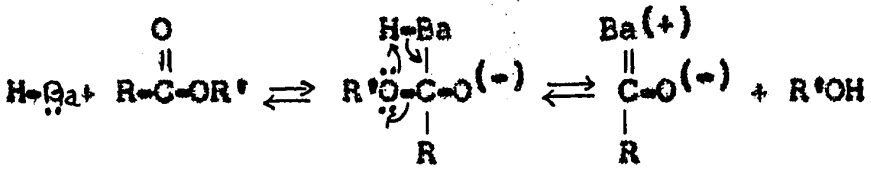
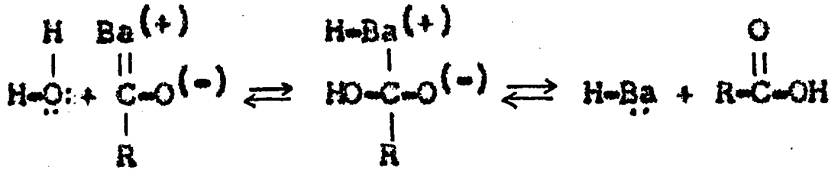


Fig. 9 Schematic representation of the interaction of ACh and inhibitors with the active site of AChE. The active site is composed of one or two anionic sites and the esteratic site containing an acidic group (Ac) and a basic group (Ba). In the figure are demonstrated the interactions of AChE with ACh (A) and with decamethonium (B). The structure of AChE after its reaction with dipropyl phosphofluoridate and 2-trimethyl ammonium ethyl methylphosphorofluoridate iodide are represented in C and D respectively.



(A) (B)



(B) (C)

The alcohol is eliminated from the enzyme substrate complex by an electronic shift and as a result of the first phase, an acetylated enzyme is formed. This reacts with H_2O to form acetate, thus regenerating the enzyme.

The active site is not necessarily a part of the molecule which when isolated would be capable of simulating the properties of the complete enzyme; its configuration as occurring within the intact native protein molecule depends, apart from its amino acid composition and sequence on the secondary and tertiary structure of the latter. It is this subtle configuration that is required for complete enzymatic activity.

This is lost when isolation withdraws the active site from the influence of those forces that prevail in the intact native molecule.

Fig.9, p. 34 gives a representation of the interaction of ACh and inhibitors with the active site of AChE[34].

II. Molecular Mechanism for Hydrolytic Enzyme Action

Kinetic Studies[6]

From studies of hydrolysis catalysed by enzyme, several important conclusions have emerged. One of these, coming particularly from pH studies, is that both an acid (A-H) and a basic (-B) group are in some way involved in the reaction. A second conclusion is that there are two reaction intermediates, an addition (Michaelis) complex (ES) and an acylated enzyme (ES'), so that the process may be represented as:

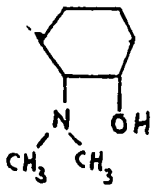
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2$$

The site that becomes acylated has been a matter of some controversy, both imidazole and serine being possibilities.

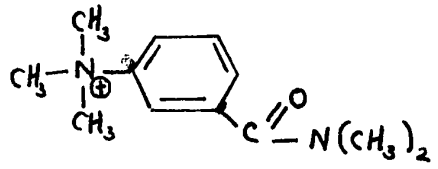
Laidler and Krupka[35] carried out a series of investigations on AChE based on the effects of pH changes and of various inhibitors. On the basis of a kinetic analysis they arrived at certain conclusions relating to the detailed configuration of the active centre of the enzyme and to the way in which the substrate and inhibitors interact with the various components of the active centre.

By working with two substrates, for one of which $k_2 > k_3$ and for the other $k_3 > k_2$, it has proved possible to separate effects on ES from those on ES'. Proceeding

in this way, so far as pH changes were concerned, Laidler and Krupka found that the basic group on the free enzyme had a pK of 6.5 and the acid group one of 9.3. In the Michaelis complex ES the basic group was no longer able to add on a proton at pH 6.5, but in the acyl enzyme ES' the proton could be added on at about this pH. The group of pK 6.5 was believed to be the imidazole group, and Laidler concluded that this group interacts with the carbonyl group of the substrate in the Michaelis complex, but does not in the acyl enzyme. There must therefore have been a transfer of the carbonyl group to another group, presumably serine, during the process ES $\longrightarrow$ ES'.



I



II

Laidler and Krupka also concluded that when non-competitive inhibitors (type I) became attached to the free enzyme they did so at the anionic site and at the acid (A-H) site, a hydrogen bond being formed between the hydrogen of the acid site and the oxygen atom on the inhibitor. The presence of the substrate prevented a similar attachment in ES, but since in ES' the acyl group of the substrate had become transferred to serine a two-point attachment of the inhibitor was now possible. Since deacetylation was now

blocked, the hydrogen atom of the acid site must have been required for this process.

In competitive inhibitors (type II) the attachment to the free enzyme was at the anionic site and at the imidazole nitrogen, which formed an electrostatic bond with the C=O dipole of the inhibitor. Again, no attachment to ES was possible. For ES' Laidler concluded that attachment was only at the anionic site; the acyl group on the serine prevented attachment at the imidazole nitrogen. Since the inhibitor did not interact with the hydrogen atom of the acid site there was no inhibition of acetylation.

B. SECONDARY DEUTERIUM ISOTOPE EFFECTS IN RELATION TO THE STRUCTURE OF THE MICHAELIS COMPLEX OF AChE

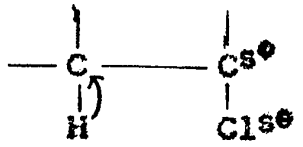
As mentioned in the general introduction, Wilson stressed the possibility of a change in the hybridization of the carbonyl carbon in the Michaelis complex; it would be transformed into the tetrahedral configuration. Of considerable interest in this connection is an old literature report by H. Erlenmeyer and Lobeck[36] who observed the operation of an isotope effect when ACh was labelled with deuterium in the acetate methyl group. These authors claimed that this deuterated ACh is 30% less active as a cholinomimetic.

Beta-deuterium isotope effects have been reviewed extensively, e.g. by Bigeleisen[38], Shiner[37,40] and Melander[39],

so that only a general outline of the most pertinent examples will be given. A secondary deuterium isotope rate effect is defined as an effect on the rate of reaction caused by the substitution of deuterium for a hydrogen that is not being transferred in the rate controlling step. Most of the important isotope rate effects indicate that there exists some intramolecular interaction between the reaction site and the site of isotopic substitution. This interaction is witnessed by energy changes in the transition state of the species involved in the reaction.

Shiner[41] observed beta-deuterium isotope effects in solvolyses of tertiary alkyl chloride (ref. Table 2, p.40). The substitution of deuterium for beta-hydrogens leads to lower rates of reaction. It is believed[42] that the rate-determining step of the reaction involves breaking of the chlorine carbon bond into the ionic fragments, chloride and carbonium ions.

In the transition state, the electron deficient reaction site is better stabilized through hyperconjugation by electron release from a beta C-H than it is from a beta C-D bond.



(1)

The opposite effect may also be observed: a substance which is electron deficient with respect to the transition state should show a secondary isotope effect in the opposite direction to the one operative in S_{N1} solvolyses.

Table 2

Tertiary Alkyl Chloride Solvolyses

80 per cent Aqueous Alcohol, 25°C

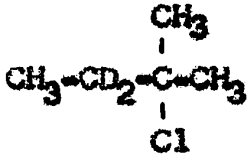
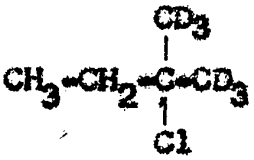
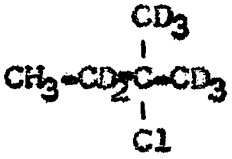
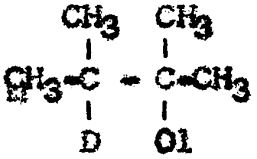
	
k_H/k_D 1.40	k_H/k_D 1.77
	
k_H/k_D 2.35	k_H/k_D 1.28

Table 3

Alkaline Hydrolysis of Ethyl Acetate

$CH_3COOC_2H_5$	$R_H = 11.4 \pm 0.15 \times 10^{-2}$ l mole^{-1} sec^{-1}
$CD_3COOC_2H_5$	$R_D = 12.7 \pm 0.18 \times 10^{-2}$ l mole^{-1} sec^{-1}
	$R_H/R_D = 0.896 \pm 0.017$

Bender[43] measured the alkaline hydrolysis of ethyl acetate (cf. Table 3, p.41). An increase in rate was observed with deuterated ethyl acetate. The hydrolysis of ethyl acetate is believed to proceed through the rate determining formation of a tetrahedral intermediate. This assumption provided a further incentive for the application of secondary beta deuterium isotope effects to the hydrolysis of acetylcholine especially since Wilson postulated that a tetrahedral structure would be involved in the Michaelis complex.

C. QUANTITATIVE EVALUATION OF LONDON-VAN DER WAALS DISPERSION FORCES

The special role played by the acetate methyl group of acetylcholine stimulated our interest in the quantitative determination of Van der Waals forces between a methyl group and hydrocarbon chains.

Dispersion forces arise even between neutral non-polar molecules and are due to the average interaction of an instantaneous electric moment on one molecule, brought about by charge density fluctuations, and the moment it induces in the other molecule. Such forces will be effective between all groups of lipid character and protein chains, but they will be specially important in the interaction of non-polar groups since they are the only forces which can contribute positively to adsorbtion.

Lionel Salem[44] considered in great detail the London-Van der Waals forces between lipid chains, and in particular between the long hydrocarbon tails of fatty acids, triglycerides, and phospholipids. Salem obtained an expression for the dispersion energy of interaction between two parallel and opposed hydrocarbon chains belonging to two different lipid molecules (cf. Fig.10, p.64) as a function of their mutual length L and of the distance D separating them. The dispersion energy between the two systems was found to be locally-additive[45]. The total interaction energy could be calculated by adding up the forces between pairs of atoms and bonds.

This concept is illustrated[46] in the model shown in Fig.10, p. 64. A convenient basic unit is a $\overset{H}{C}-C$ group centered in the middle of the C-C bond; each carbon atom belongs to two units, so that the unit is equivalent to a CH_2 group. Salem arrived at the general expression (5) which yields the total dispersion force between two CH_2 groups:

$$W_{disp_{CH_2 \leftrightarrow CH_2}} = \frac{A \cdot 3\pi N}{8 \, l \, D^5} \qquad (5)$$

A is a constant in atomic units and is obtained by adding the nine effective bond-bond dispersion interactions between two singly opposed CH_2 units (ref. Fig.10, p.64).

$W_{CH_2 \leftrightarrow CH_2} = W_{CC \leftrightarrow CC}$(8.20 a.u.) + 4 $W_{CC \leftrightarrow CH}$(37.94 a.u.) + 4 $W_{CH \leftrightarrow CH}$(48.80 a.u.) (ref.Table 4,p.45) A = 96.94 a.u.

The total dispersion energy $W_{disp}\ CH_2 \longleftrightarrow CH_2$ between two singly-opposed CH_2 units (N=1) was equal to 0.4 kilocalories per mole at D = 5°A. Hence the total energy is proportional to the number of units in each of the interacting molecules, and also proportional to the inverse fifth power of the intermolecular distance. One may therefore expect rather large forces of attraction between long hydrocarbon chains, and also a very sensitive variation of these forces with distance, even when the molecules are in close contact. Eg. if two chains, previously 5 angstroms apart, come 1 angstrom closer, the total attraction would be tripled.

Table 5, p. 45 gives the dispersion energies between the hydrocarbon tails of neighbouring fatty acids in monomolecular films. The feature to be noted is the large difference in the attractive energy between a stearic acid molecule and an isostearic acid molecule. The presence of the dimethyl group in isostearic acid reduces the total attraction by a factor of 3. This is an outstanding example of the great sensitivity to distance of dispersion forces. These forces are therefore highly distance-specific; they can increase from negligible values to large values if a minor structural change enables the two molecules to come into closer contact.

Table 4

Bond-bond Dispersion Interaction Energies*

Bonds	C - H sp^3	C - C sp^3 sp^3	C = C sp^2 sp^2
C - H sp^3	12.20	9.985	27.27
C - C sp^3 sp^3	4.985	8.200	22.20
$C_{sp^2} = C_{sp^2}$	27.27	22.20	61.55

* (Coefficient of d^{-6} in atomic units; all coefficients are negative; the number in the table refers to the interaction of the two bonds in the corresponding row and column[46]).

Table 5

London-Van der Waals Dispersion Energies in Monolayer Films

(attraction between nearest neighbour hydrocarbon tails).

Formula	Name	D(A)	N	$W_{attr.}$(Kcal/mole)
$CH_3-(CH_2)_{16}-COOH$	Stearic Acid	4.8	-18	-8.4
$CH_3-(CH_2)_{34}-COOH$	Hexatriacontanoic Acid	4.8	-36	-16.8
$CH_3-CH-(CH_2)_{14}-COOH$ CH_3	Isostearic	6.0	-18*	-2.8

* The slight decrease in effective chain-length due to the iso-CH_3 group is neglected[47].

D. THE ROLE OF HYDROPHOBIC INTERACTIONS IN THE FORMATION OF ENZYME-SMALL MOLECULE COMPLEXES

A factor affecting the forces of interaction between enzymes and substrates and which appears to have been entirely ignored in the past, consists in hydrophobic interactions between non-polar residues and the solvent water. It is generally agreed that non-polar groups cause local disturbances of the hydrodynamic properties of water with the result that the first few layers of water molecules in closest contact with the hydrophobic group are more polarized and more tightly bound to each other. As a consequence, the entropy of the solvent water decreases and if a mechanism is made available for the transfer of the non-polar group to a non-aqueous medium, an increase in the entropy of water will result. It follows that the transfer of a non-polar group from water to a hydrophobic environment will benefit from a considerable driving force and since enzymes can provide non-polar regions of binding or adsorption, it is clear that hydrophobic interactions alone will favor at times the adsorption of small molecules carrying suitable substituents. It can be seen here that no positive contribution of Van der Waals forces to binding can be operative if the transfer process is visualized as a dissolution of a non-polar group into a non-polar region of a protein.

Quantitative evaluations of hydrophobic interactions

between hydrocarbon residues and water have been made by Cohn and Edsall[48] who worked out a simple technique based on solubility measurements. In order that the activity coefficients of the molecules approach unity as much as possible, compounds of very low solubility in water and non-polar solvents were selected. The amino-acids proved suitable for such studies.

Relative Solubilities in Water and in Organic Solvents As a Measure of Hydrophobic Interactions

The determination of relative solubilities in water and in other solvents provides data for determining the free energy of transfer of a solute from one medium to another. An ideal experiment is considered, in which water and the organic solvent under consideration are separated by a semi-permeable membrane, impermeable to both solvents but permeable to the solute, which is present at infinite dilution. At equilibrium, the activity of the solute, a, in both phases must be identical. Denoting its mole fraction in the aqueous phase by N_W^o, and in the organic solvent by N_A^o, and the corresponding activity coefficients by f_W and f_A respectively, we have:

$$a = f_W N_W^o = f_A N_A^o \qquad (1)$$

Since an infinitely dilute solution of the solute in water is taken as the standard state, $f_W = 1$ by definition, and

$$f_A = N_W^o / N_A^o \qquad (2)$$

Now let us look at a saturated solution of the solute in water (at mole fraction N_W), and in the organic

solvent (at mole fraction N_A). If the same solid phase is in equilibrium with both saturated solutions, then the activity of the solute in both saturated solutions, a_s, must be the same as its activity in the solid phase.

$$a_s = N_W f_W(\text{sat.}) = N_A f_A(\text{sat.}) \qquad (3)$$

Amino acids are relatively insoluble in all organic solvents, so insoluble that we may generally assume that the activity coefficient is independent of the mole fraction of amino acid in such solvents; that is, that f_A in equation 2 equals f_A(sat.) in equation 3. Thus the activity coefficient of an amino acid in any organic solvent is given by its solubility ratio in water and that solvent, multiplied by its activity coefficient in the saturated aqueous solution.

$$f_A = \left[\frac{N_W}{N_A}\right]_{(\text{sat})} f_W(\text{sat}) \qquad (4)$$

The free energy of transfer of the amino acid from a dilute aqueous solution (at mole fraction N_W) to the solvent A (at mole fraction N_A) is given by the relation:

$$F_A - F_O = RT \ln f_W + RT \ln \frac{N_A}{N_W} \qquad (5)$$

The values of f_W(sat) in water are generally not widely different from unity. In the following discussion, therefore, we shall frequently speak of the solubility ratios as if they gave directly the activity coefficient of the amino acid in the organic solvent, although strictly this is true only as a first approximation. (The non-ideality

of our solutions of inhibitors will play an important role in the interpretation of results obtained).

Effect of the CH_2 Groups of Solutes on Solubility Ratios

The solubility in water of the monoamino-monocarboxylic acids, from glycine to leucine and norleucine, decreases with increasing number of CH_2 groups in the hydrocarbon chain.

If solubility ratios in water and organic solvents are considered, the effect of the CH_2 group is perfectly regular. The influence of each CH_2 group in the transfer from water to ethanol appears to be the same for the alpha-amino acids or for their derivatives. This may be demonstrated on the basis of such data as those in Table 6, p.50 by subtracting log N_A/N_O (where N_A is the solubility in mole fraction in ethanol) for glycine from that for alanine and for alpha-aminobutyric acid. The differences per CH_2 group are nearly the same, not only for these substances, but for other amino acids of the same family that have been adequately studied as well as for derivatives of the amino acids and for their isomers, such as the alpha-hydroxyamides, glycolamide and lactamide. The increment in log N_A/N_O per CH_2 group is always positive, and is approximately equal to 0.49. In other words each CH_2 group in side chains terminating in a methyl group may be thought of as increasing solubility in ethanol relative to that in water three-fold. In addition, it could be shown that the nature

Table 6

Influence of Structure on Solubility Ratio in Water and Ethanol at 25°C

Substance i and k	$(\log N_A/N_W)_i$	$(\log N_A/N_W)_k$	$\frac{\Delta \log N_A/N_W}{\Delta n}$
	Influence of CH_2 group		
Glycine-alanine	-3.391	-2.856	+0.54
Glycine-alpha-aminobutyric acid	-3.391	-2.375	+0.50
Glycine-alpha-aminocaproic acid	-3.391	-1.414	+0.49
Valine-leucine	-2.158	-1.622	+0.54

Data taken from Cohn and Edsall[48].

Table 7

Influence of the Structure of the Solvent upon the Solubility Ratio in Water and Organic Solvents at 25°C

Group of Solute	Organic Solvent	log N_A/N_W	$\Delta F_{(calculated)}$*
CH_2	Methanol	0.46	600
	Ethanol	0.49	668
	Butanol	0.53	723
	Heptanol	0.53	723
	Acetone	0.49	668

* Change in free energy with change in solvent in calories per mole.

Data taken from Cohn and Edsall[49].

of the non-aqueous solvent is without significant effect on the free energy of transfer of CH_2 groups (cf. Table 7, p.51).

FOREWORD TO EXPERIMENTAL PART

To gain some insight into the stereochemical properties of the Michaelis complex between (ACh) and (AChE, through the application of secondary deuterium isotope effects, four deuterated ACh isomers were synthesized and their Michaelis constants determined.

A detailed study of the interaction of the acetate methyl group of ACh with AChE was undertaken. This was made possible by the availability in our laboratories of a wide variety of quaternary salts of the 1-3 dioxalane series. This also led to the synthesis of formyl choline and to solubility studies.

EXPERIMENTAL

The Synthesis and Configuration of Quaternary Salts in the 1,3-Dioxolane Series

The quaternary salts in their d,l form were synthesized by Dr. Bernard Belleau and Dr. D.J. Triggle[50].

L(+) and D(-)-cis-2-methyl-4-trimethylammonium methyl 1,3-dioxolane iodide were obtained by Dr. Bernard Belleau and Dr. J. Puranen[51].

Synthesis of cis 2 methyl^{D_3}-4 hydroxymethyl 1-3 dioxolane. It was obtained using the method of B. Belleau and Triggle[52] but substituting deuterium gas for hydrogen gas, D_2O for H_2O, CD_3OD for CH_3OH and Na_2CO_3 for $NaHCO_3$ in the synthetic procedure.

Synthesis of 4 Deuterated Isomers of Acetylcholines

1' $CD_3COOCH_2CH_2\overset{\oplus}{N}(CH_3)\overset{\ominus}{Br}$ was prepared according to the method of H. Erlenmeyer and H. Lobeck[53].

$$CD_3COOD \xrightarrow{SOCl_2} CD_3COCl \xrightarrow{\begin{matrix}OH\ \ Br\\ |\ \ \ |\\ H_2C\text{-}CH_2\end{matrix}} \quad (a)$$

$$CD_3COOCH_2CH_2Br \xrightarrow{(CH_3)_3N} CD_3COOCH_2CH_2\overset{\oplus}{N}(CH_3)_3\overset{\ominus}{Br} \quad (b)$$

Procedure:

a) Acetyl-d_3 Chloride. 0.77 g (.0012 Eq) deuterated acetic acid was treated with slight excess -- 1.75 g (.015 Eq)-- of thionyl chloride. The deuterated acetyl-chloride distilled at 47-51°C.

b) 2-Bromoethyl Acetate-d_3. A solution of .97 g (0.012 Eq) acetyl-d_3 chloride in dry ether was treated with an ether solution of 1.6 g (0.013 Eq) of ethylene bromohydrin and 0.95 g (0.012 Eq) of pyridine. Water was added; the ether layer was washed with dilute sulfuric acid and with a solution of sodium carbonate and dried over sodium sulphate. After the removal of ether the 2-bromoethyl acetate-d_3 was distilled. b.p. 81-82°.

c) Acetyl-d_3-choline bromide. According to the procedure of Fourneau and Page[54], 2 g (0.012 Eq) 2-bromoethyl acetate-d_3 was treated with a solution of trimethylamine in dry benzene in a pressure bottle at 100°C. The reaction was completed after 4 hours and the resulting acetyl-d_3-choline bromide was twice recrystallized from absolute ethanol. Yield 2.35 g (0.010 Eq), 87%, m.p. 143-144°C.

2' $CH_3COOCH_2CH_2\overset{*}{N}(CD_3)\overset{*}{Br}$, method of V. du Vigneaud[55].

$$CD_3Br \xrightarrow{HOCH_2CH_2NH_2} HOCH_2CH_2\overset{*}{N}(CD_3)_3\overset{*}{Br}$$

Procedure:

a) 5 g of methyl-Cd_3 iodide was added (exothermic reaction) to a test tube containing 3.75 g of ethanolamine and cooled in an ice-salt bath. The mixture was kept at room temperature with occasional shaking for 60 hours. The contents of the test-tube were then dissolved in 25 ml of 0.2 N sodium hydroxide and to the solution was added

275 ml of a saturated aqueous solution of ammonium reineckate (ammonium salt of Reinecke's acid, tetrathiocyanodiammonochromic acid), $NH_4[Cr(NH_3)_2(SCN)_4].H_2O$. The mixture was cooled overnight to effect complete precipitation of d_9-choline reineckate. It was collected and dried; the d_9-choline salt weighed 5.75 g (35% yield).

The salt was decomposed with Amberlite IRA 401 - Bromide form. The reineckate was absorbed on a column of the resin and eluted with water. The clear filtrate was then evaporated to dryness at 35-40° in vacuo. The residue was extracted with absolute alcohol, the solution was filtered and again concentrated to dryness. The d_9-choline bromide weighed 1.725 g.

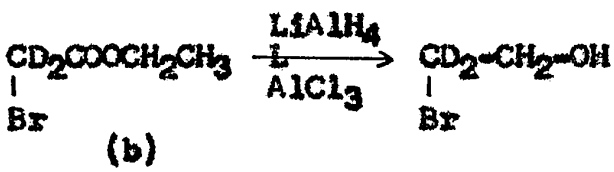
$$HOCH_2CH_2\overset{\bullet}{N}(CD)_3\overset{\bullet}{Br} \xrightarrow{Ac_2O} \overset{\bullet}{Br}(CD_3)\overset{\bullet}{N}CH_2CH_2OOCCH_3$$

b) The d_9-choline bromide[56] was refluxed with 25 ml of acetic anhydride under an atmosphere of nitrogen. After removal of the excess anhydride, the yield was 0.275 g (86%) of slightly colored product, m.p. 143-144° (sintering at 140°). Recrystallization from ethanol-ether gave a pure product melting at 143-144°C.

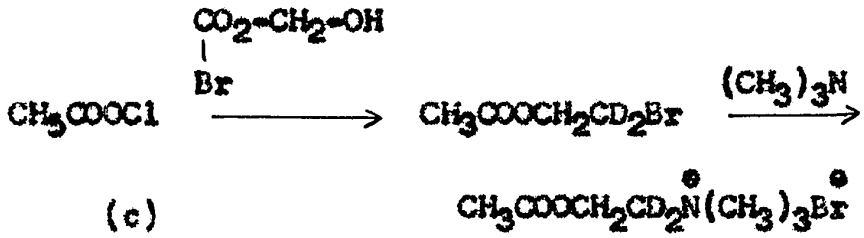
3' <u>$CH_3COOCH_2CD_2\overset{\bullet}{N}(CH_3)_3\overset{\bullet}{Br}$:</u>

$$CD_3COOD \xrightarrow[(P)]{Br_2} \underset{\underset{Br}{|}}{CD_2COOBr} \xrightarrow{CH_3CH_2OH} BrCH_2COOCH_2CH_3$$

(a)



(b)



(c)

Procedure:

a) Ethyl d_2-bromoacetate: was prepared by the method of Answer and Berniardi[57]. To a mixture of 5 g of deuterated acetic acid and 0.5 g of red phosphorous was added gradually 60 g of dry bromine with cooling. The mixture was heated under reflux for 15 hours on a water bath, then treated slowly with 2-3 times the calculated amount of absolute ethanol. The mixture was diluted with water, and the product was extracted into ether, which was washed with a sodium bicarbonate solution, dried and fractionally distilled to give ethyl d_2-bromoacetate, b.p.$_{20}$ 80-81°. Yield 4.6 g (85%). C-D stretching band in IR at 2160 cm^{-1}.

b) Ethylene d_2-bromohydrin: 4.6 g of ethyl d_2-bromoacetate was added to a mixture of 1 g of lithium aluminum hydride and 5.3 g of aluminum trichloride in 50 ml of dry ether. The mixture was refluxed for 2 hours. The resulting mixture was decomposed with water, the salts filtered off, the ether evaporated and the ethylene d_2-bromohydrin purified by vacuum distillation: b.p. 85-6°. Yield 20 g (55%). Infra-

red C-D stretch at 2160 cm^{-1}.

c) The acetyl-d_2-choline was prepared as described above in the case of acetyl-d_3-choline.

4' $CH_3COOCD_2CH_2\overset{\bullet}{N}(CH_3)_3\overset{\bullet}{Br}$ 56

$$CH_3COOH \xrightarrow[(P)]{Br_2} CH_2(Br)COOBr \xrightarrow{CH_3CH_2OH} BrCH_2COOCH_2CH_3$$

(a)

$$Br\text{-}CH_2COOCH_2CH_3 \xrightarrow[AlCl_3]{LiAlD_4} CH_2(Br)\text{-}CD_2\text{-}OH$$

(b)

$$CH_3COOCl \xrightarrow{CH_2(Br)\text{-}CD_2\text{-}OH} CH_3COOCD_2CH_2Br \xrightarrow{(CH_3)_3N} CH_3COOCD_2CH_2\overset{\bullet}{N}(CH_3)_3\overset{\bullet}{Br}$$

(c)

Procedure:

Prepared as above (3') but substituting acetic acid by d_3-acetic acid and $LiAlD_4$ by $LiAlH_4$.

Synthesis of Formyl Choline

$$HCOOH + HOCH_2CH_2Br \xrightarrow[-H_2O]{\text{dry } C_6H_6} HCOOCH_2CH_2Br$$

(a)

$$HCOOCH_2CH_2Br \xrightarrow{N(CH_3)_3} HCOOCH_2CH_2\overset{\bullet}{N}(CH_3)_3\overset{\bullet}{Br}$$

(b)

Procedure:

a) 2-Bromo ethyl formate: 11.5 g of 98% formic acid was placed in a flask containing 31 g of ethylene bromohydrin and 150 ml of dry benzene. The mixture was refluxed for five hours using a Dean-Stark water trap to entrain the water as it is produced. The benzene was evaporated and the product dried over 50% sodium sulfate and then purified by distillation. Yield, 20 g. Ethyl bromoformate: b.p. 75-76°/90mm. Carbonyl band at 1710 cm^{-1}.

b) Formyl Choline Bromide was obtained in the conventional manner as follows: 20 g ethyl bromoformate was reacted with an excess of trimethylamine in dry benzene in a pressure bottle at 100°C for four hours. Yield 25 g (96%) of formyl choline bromide, m.p. 147-149°C. Carbonyl band at 1700 cm^{-1} (mull).

The product was purified by recrystallizing twice from absolute ethanol. The final purity of the product was determined through its saponification equivalent (93% of theoretical value) and a micro-determination for ionic bromine; Calculated: Br^-, 37.67%; Found: Br^-, 37.82%.

Methods

1- Techniques for the Determination of Inhibitor Constants With the System AChE-ACh

The procedure for measuring rates consisted in the electrometric titration method described previously by Stein and Laidler[58] (1959), use being made of a pH-stat manufactured

by Ole Dich of Copenhagen. The measurements were carried out at 24.95 ± 0.03°C with 25 ml of the reaction mixture containing 0.04 M $MgCl_2$ and 0.05 M NaCl, in addition to the enzyme and substrate. During the course of the reaction a slow stream of CO_2-free nitrogen was passed over the surface of the solution in order to minimize the effects of CO_2 absorption. In addition, the reaction vessel was topped with a teflon head especially fitted to accommodate the electrodes, syringe, pipette and nitrogen inlet tube. This arrangement was found to eliminate the effects of CO_2. solution at pH 7.4.

The acetylcholinesterase was obtained from a commercial source (Nutritional Biochemicals), the source being bovine erythrocytes. A dilute solution of the enzyme in isotonic solution at pH 7.4 and possessing a specific activity of approximately 3 international units was prepared [1 enzyme unit being defined as the enzymic activity which under the conditions in the present case, (25.1°C, pH 7.4) can decompose 1 micromole of acetylcholine per minute per ml of enzyme solution]. The solution was kept at 0°C in ice-water and a 200 µl aliquot was taken for each run. The acetylcholine bromide was a pure preparation supplied by Eastmann Kodak Ltd.

The hydrolysis of ACh was studied at pH 7.4. The substrate concentrations varied from 1.3×10^{-4} to 1.3×10^{-3}M; the titrating solution of sodium hydroxide was 0.020 N. Before each set of runs, blanks were run so

that a correction could be made for CO_2 absorption and for non-enzymatic hydrolysis; these effects were not significant at the pH value of 7.4.

2- Assay for Acetaldehyde

The procedure was a modification of Colowick and Kaplan[59] assay.

a) Reagents

Isotonic solution: 0.04 M in $MgCl_2$ and 0.05 M in NaCl.

D.N.P. Reagent: 0.1% by weight of dinitrophenyl-hydrazine dissolved in 2N HCl.

Enzyme Solution: as supplied by NBC.

Acetaldehyde: reagent grade; a dilute solution containing a known weight by volume was made to approximately 5×10^{-4}M.

b) Incubation with AChE

20.0 mg samples of cis-2-methyl-4-dimethylamino-methyl-1,3-dioxolane methiodide were dissolved in 5.0 cc of isotonic solution at pH 7.4. To one solution was added 50 μ l of enzyme solution (using a micro-syringe) containing approximately 10 units of enzyme activity. The solutions were incubated at 25.0°C in a constant temperature bath.

After time τ, a 0.1 cc aliquot was taken out and made alkaline to pH 10 with 0.2 g K_2CO_3. The aliquot was steam distilled in a micro-Kjeldahl apparatus until approximately 5 cc of distillate was collected.

c) Extraction of Acetaldehyde

To the 5 ml portion of distillate was added 1 ml of D.N.P. reagent. The resulting solution was extracted with 5 ml of CCl_4 (shaking time of 5 minutes). The CCl_4 extract was washed four times with 2 ml portions of water. The extracts were dried over Na_2SO_4.

d) Colorimetric Assay:

The CCl_4 extract was decanted and transferred to a cuvette. The optical density was read on a Bausch and Lomb Spectronic Model 20 at 420 mμ using matched cuvettes. Enzyme blanks and standards were carried through the same procedure. The concentration of acetaldehyde was read directly from a standard curve.(ref. Fig.15, p.70).

3- Automatic Electrometric Determination of Bromide in Formylcholine Bromide

a) Principle:

Bromide ion in dilute acetic acid solution is potentiometrically titrated with $AgNO_3$, using a silver electrode as an indicator. The titration is automatically stopped at the end-point by means of a magnetic valve operated by an Ole Dich titrator.

b) Reagents:

0.02000 M $AgNO_3$: 3.3978 g of $AgNO_3$ (A.R.) dissolved in 1 litre of distilled water. The reagent is stable for a few months if stored in an amber glass bottle.

0.1000 M NaBr standard solution: 1.0291 gram dried NaBr (A.R.) dissolved in 100 ml of distilled water.

Acetic acid solution: 50% by volume in water.

c) Determination of the End-Point Potential:

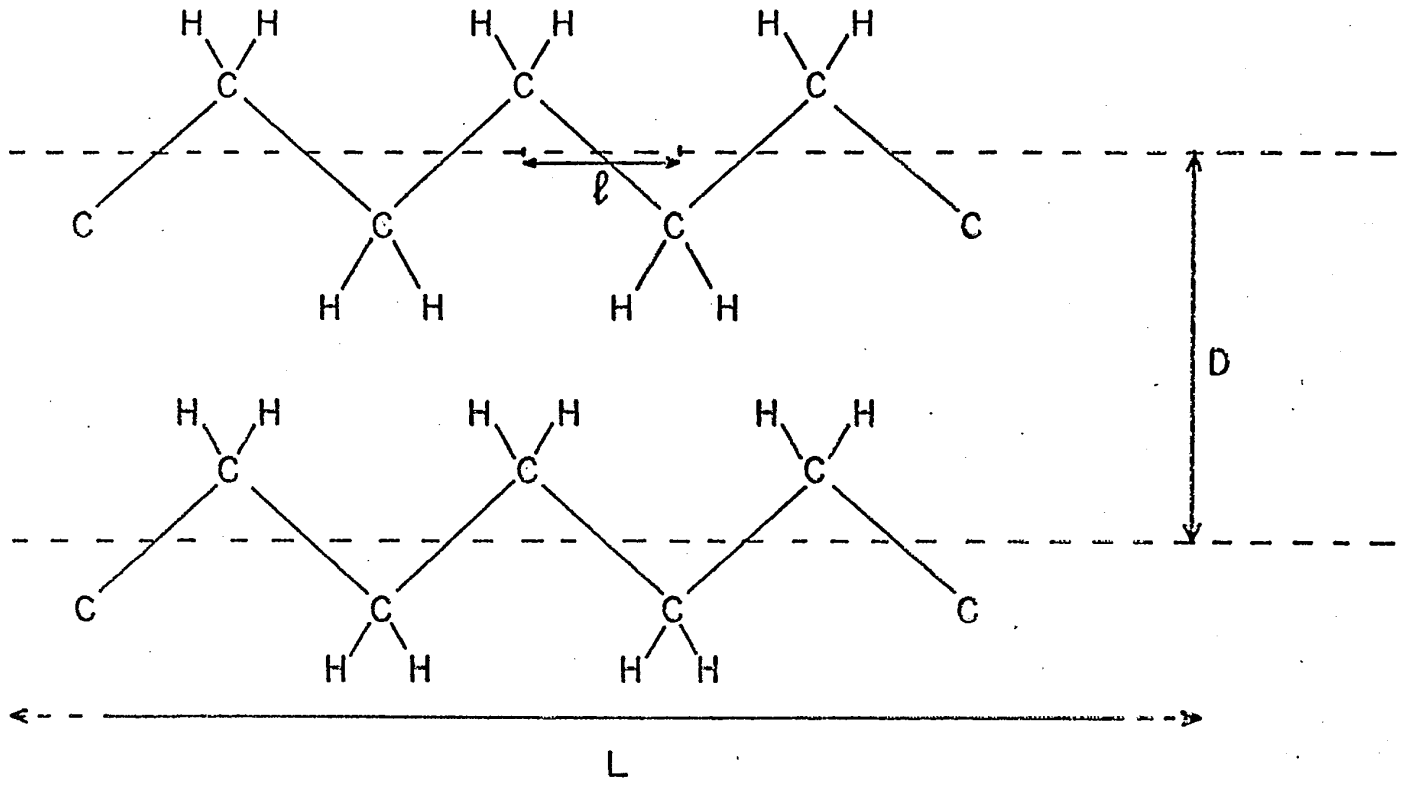
200 µl of standard NaBr solution was added to approximately 25 ml of 50% acetic acid. The end-point occurs around 100 millivolts. It can be determined accurately by plotting millivolts against microliters of reagent added. The steepest part of the curve will give the end-point.

4- Determination of Solubilities

An excess of pure solute was added to a given volume of solvent and the solute-solvent mixture allowed to reach equilibrium overnight with constant shaking. The saturated solution was then decanted, centrifuged and transferred to a small volumetric flask. The density of the solution was determined on a semi-micro balance. The amount of solute dissolved in grams per cc of solvent was established by comparing the density of the saturated solution with that of pure solvent.

Substrate	$K_m \times 10^{-4}$ mole litre^{-1} $\pm$ 0.4×10^{-4} mole litre^{-1}
$CH_3COOCH_2CH_2-\overset{CH_3}{\underset{CH_3}{\overset{\oplus}{N}}}-CH_3\,I^{\ominus}$	4.55
$CD_3COOCH_2CH_2-\overset{CH_3}{\underset{CH_3}{\overset{\oplus}{N}}}-CH_3\,I^{\ominus}$	4.25
$CH_3COOCD_2CH_2-\overset{CH_3}{\underset{CH_3}{\overset{\oplus}{N}}}-CH_3\,I^{\ominus}$	4.50
$CH_3COOCH_2CH_2-\overset{CD_3}{\underset{CD_3}{\overset{\oplus}{N}}}-CD_3\,I^{\ominus}$	4.70
$CH_3COOCH_2CD_2-\overset{CH_3}{\underset{CH_3}{\overset{\oplus}{N}}}-CH_3\,I^{\ominus}$	4.40

Table 8 Results of Michaelis Constant Determinations with acetylcholine and 4 deuterated acetylcholines.isomers.



PARALLEL OPPOSED CONFIGURATION FOR TWO PARAFFIN CHAINS

$$W_{disp.} = A \frac{3\pi}{8\ell^2} \frac{N}{D^5} = 0.4 \text{ kilocalories per mole per } CH_2 \text{ unit } (D = 5\,°A),\ (N = 1)$$

Fig.10

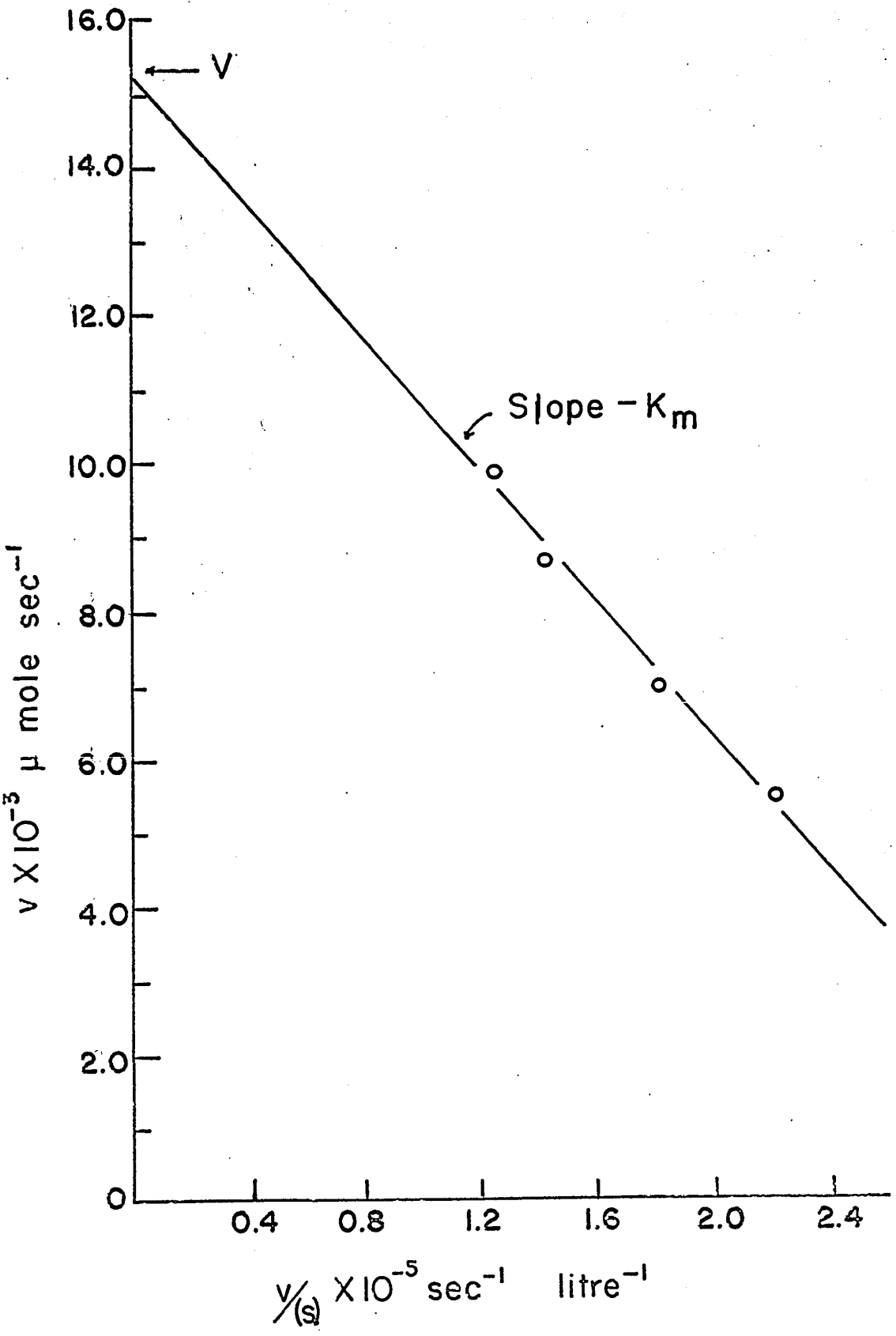


Fig. 11 Plot of v against v/(s) according to the method of Eadie for acetylcholine with bovine acetylcholinesterase.

RESULTS AND DISCUSSION

A. Secondary Deuterium Isotope Effects and AChE

The determination of beta-deuterium isotope effects on the rate of ACh hydrolysis did not provide useful information. The Michaelis constants (K_{ms}) obtained are listed in Table 8, p. 63. An illustration of the graphical method for obtaining the K_m values is given in Fig.11, p.65. Due to the rather small variations in the values obtained (±10%) and the inherent experimental error associated with those values (20%), no definite evidence of beta-deuterium isotope effects on the rate of hydrolysis on the binding of acetylcholine could be conclusively demonstrated. Pharmacological tests performed by Dr. M. Pindell and his staff at Bristol Laboratories, Syracuse, N.Y. failed to detect any isotopic effects on cholinergic receptors (the arterial blood pressure fall of the dog and on the contraction of guinea-pig ileum). This completely disproves the findings of H. Erlenmeyer[37] who reported a 30% reduction in the potency of acetyl d_3 choline bromide on leech muscle with respect to acetylcholine.

Similarly, substitution of CD_3 for CH_3 at C_2 of inhibitor III (Fig.17, p.73) in the 1-3 dioxolane series gave no significant difference in its affinity for the enzyme. The affinity constants of both deuterated and non-deuterated inhibitors (XVIII and III) may be compared in Fig. 17, p.73. The determination of the I_{50} for the

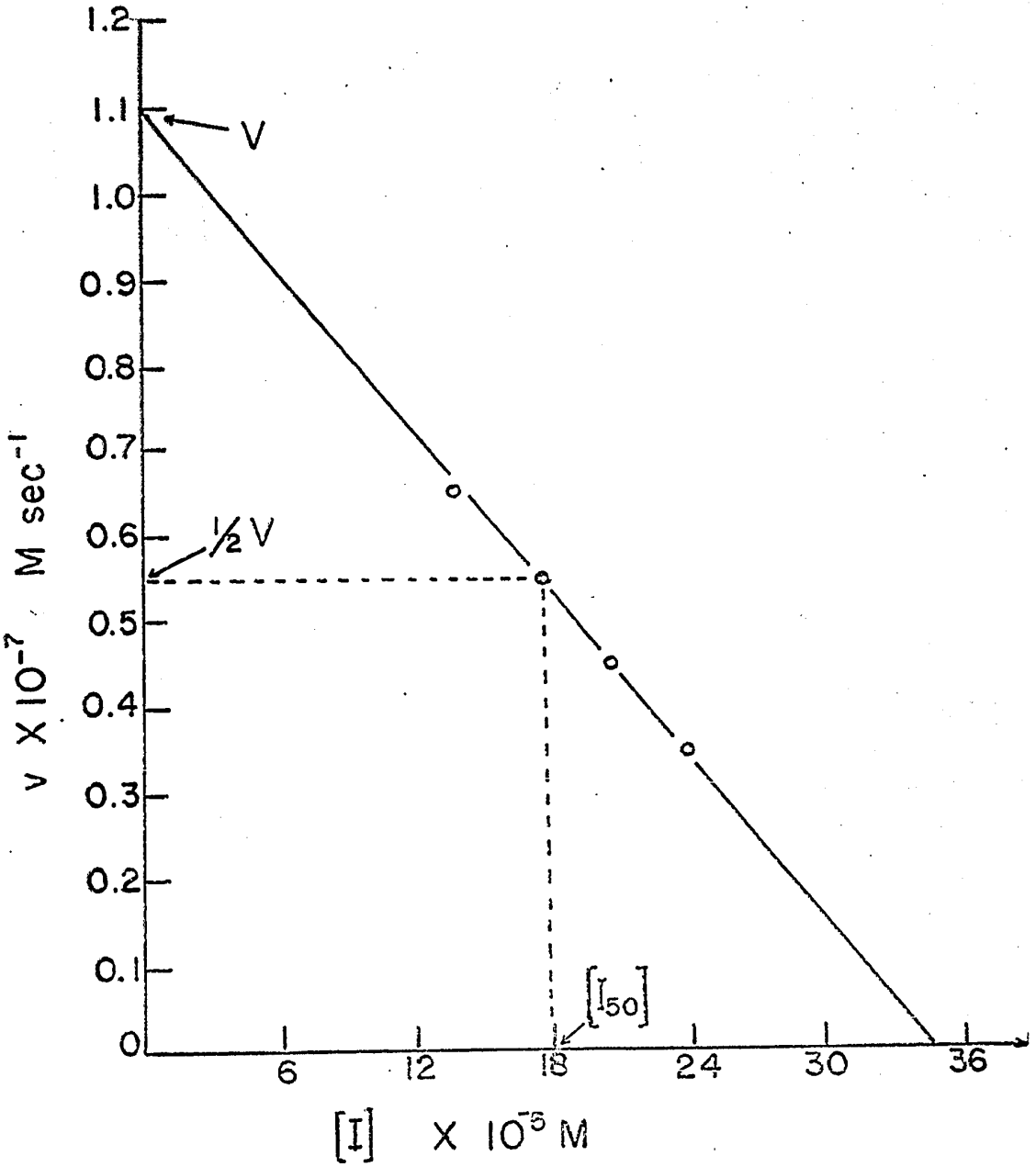


Fig. 12 Determination of the [I_{50}] for cis dl 2-methyl-4 dimethyl aminomethyl 1-3 dioxolane methiodide. [I] = 1.8 x 10^{-3}M. [S] = 1.3 x 10^{-3}M Acetylcholine Bromide.

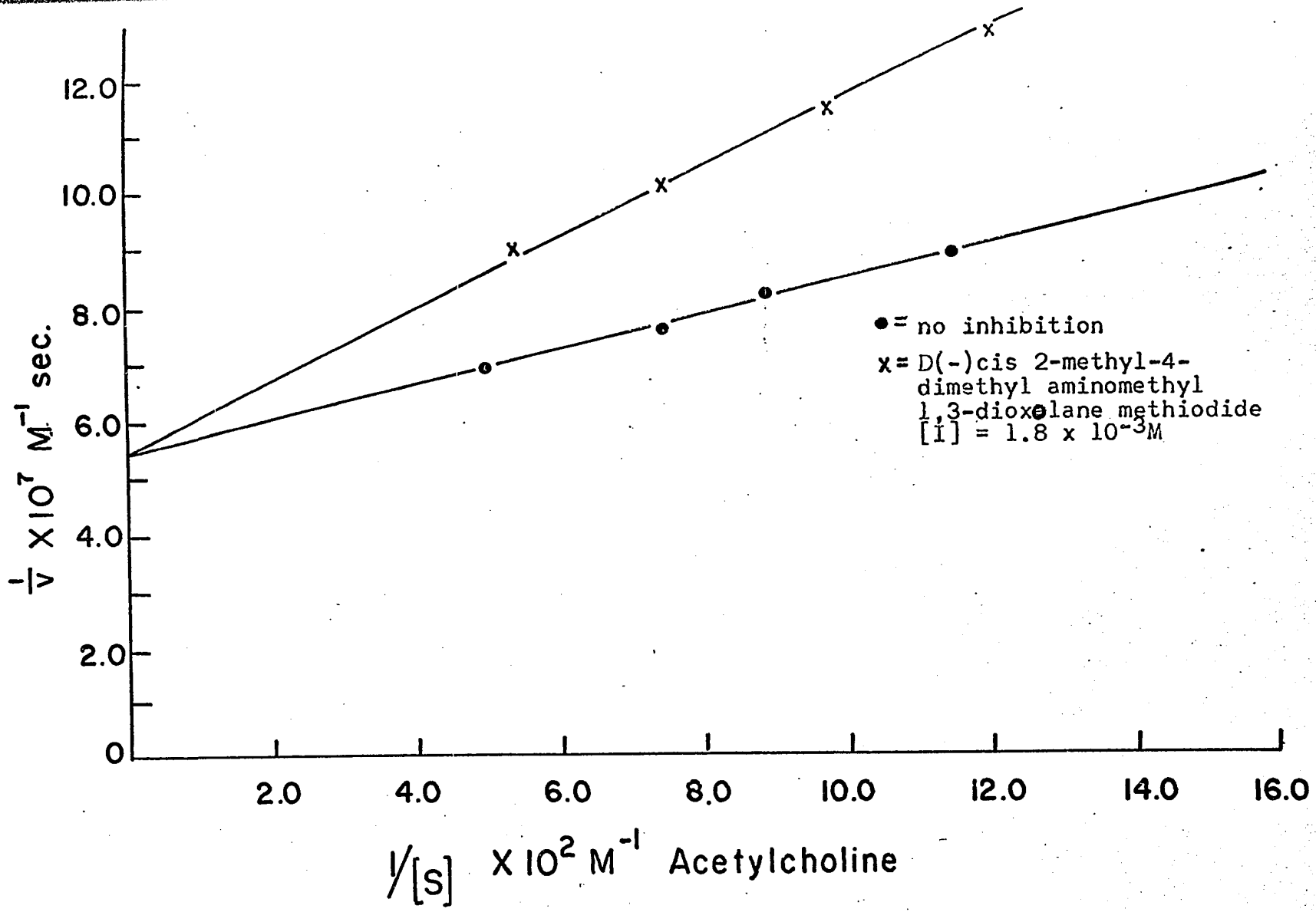


Fig. 13 Typical Determination of Competitive Inhibition for an Inhibitor.

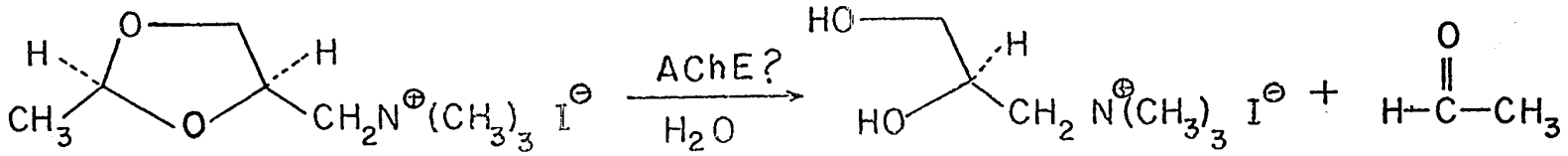


Fig. 14 Hypothetical hydrolysis of cis dl 2-methyl-4 dimethylaminomethyl-1-3 dioxalane methiodide into diol and acetaldehyde.

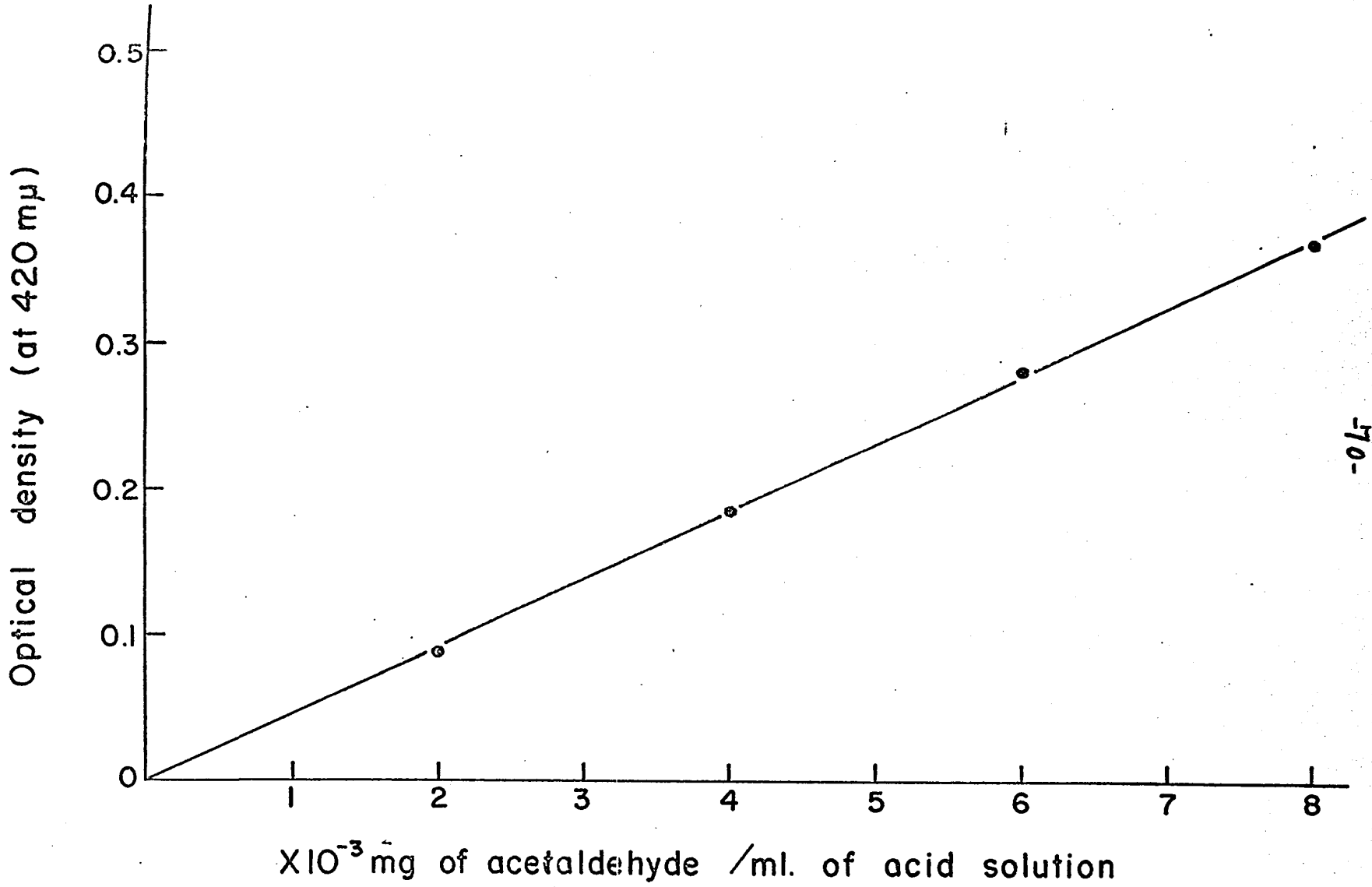


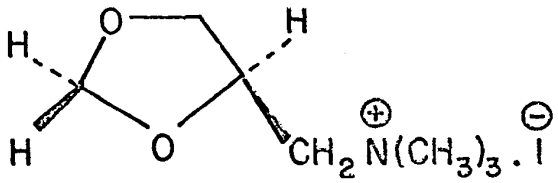
Fig. 15 Standard curve for mg. of acetaldehyde extracted into 6 ml. of 2N HCl vs. optical density using the Dinitrophenylhydrazine assay technique.

inhibitors is illustrated in Fig.12, p.67.

To prove that our inhibitors interacted with the same active site of AChE as does ACh we tested the 1-3 dioxolanes for their mode of inhibition. Fig.13, p.68 shows that typical competitive inhibition characterizes this inhibitor. In fact all the inhibitors studied were found to act competitively.

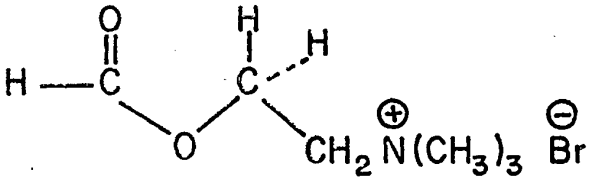
The possibility was also examined that the hydrolysis products of the inhibitors might act themselves as inhibitors (Fig.14, p.69). A micro colorimetric method for the determination of acetaldehyde was used (p.58). A graph relating optical density with the concentration of acetaldehyde is given in Fig.15, p.70. No enzymic hydrolysis of the inhibitors could be demonstrated. The corresponding diol, the expected hydrolysis product, had only a weak inhibitory activity ($I_{50} = 10^{-2}M$).

The preceding results seem to indicate that ACh may not undergo any change in the hybridization of its carbonyl group when forming an addition complex with AChE. It should be kept in mind however that the experimental limitations preclude any definite conclusions on this point. A change in hybridization would result in a positive deuterium isotope effect at least on binding (see Introduction, p.38). These results must however be interpreted with caution since the absence of secondary isotope effects could be due to the inherent limitations of the experimental technique. A small but nevertheless significant isotope



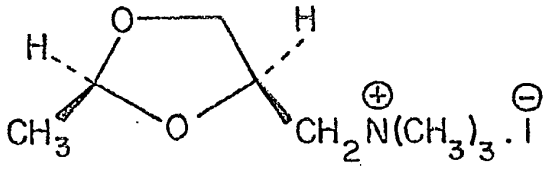
I

$K_i = 45 \times 10^{-5}$ M



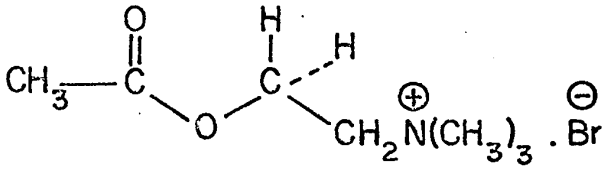
XVI

$K_m = 35 \times 10^{-4}$ M



IV

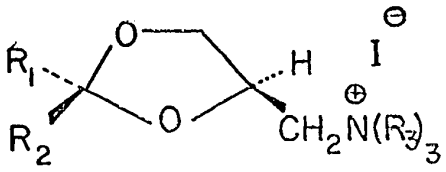
$K_i = 4.1 \times 10^{-5}$ M



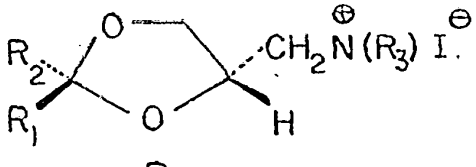
XVII

$K_m = 4.5 \times 10^{-4}$ M

Fig.16 Affinity constants of two inhibitors compared with those of acetylcholine and formylcholine.



A



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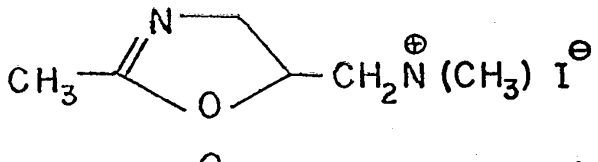
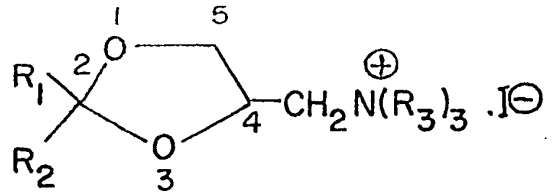


C

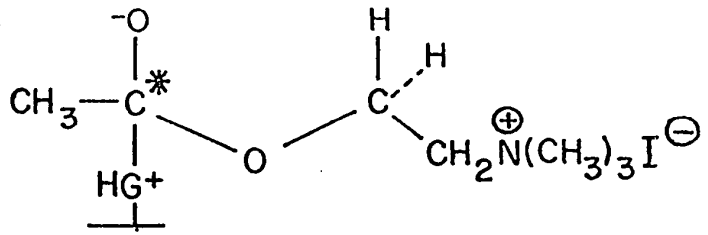
Compound No.	General Formula	Substituents R_1	R_2	R_3	Configuration and Optical Purity	K_i x 10^{-5}M	pI	Relative* Potencies	ΔF_i in Kilocalories
I	A,B	H	H	CH_3	dl	45	3.35	9	4.568
II	B	H	H	CH_3	D(100%)	50	3.30	8	
III	A,B	H	CH_3	CH_3	cis-dl	7.5	4.13	155	5.616
IV	A	H	CH_3	CH_3	cis-L(100%)	4.1	4.39	100	
V	B	H	CH_3	CH_3	cis-D(100%)	67	3.27	6	
VI	A,B	CH_3	H	CH_3	trans-dl	23	3.64	18	4.996
VII	A	CH_3	H	CH_3	trans-L(32%)	23	3.64	18	
VIII	B	CH_3	H	CH_3	trans-D(32%)	38	3.42	11	
IX	A,B	H	CH_3	C_2H_5	cis-dl	35	3.46	12	
X	A,B	CH_3	H	C_2H_5	trans-dl	40	3.40	10	
XI	A,B	CH_3	CH_3	CH_3	dl	60	3.32	7	4.520
XII	A,B	H	CCl_3	CH_3	cis-dl	1.1	4.96	370	6.761
XIII	A,B	CCl_3	H	CH_3	trans-dl	7.4	4.13	56	5.630
XVIII	A,B	H	CD_3	CH_3	cis-dl	4.2	4.37	98	
XIX	C				(oxazoline)dl	70	3.15	6	4.300

* The potency number is proportional to the affinity; the cis-L-isomer was used as the standard equal to 100.

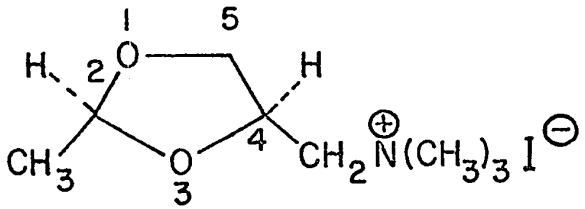
Fig.17 Potency as a function of configuration for various inhibitors.



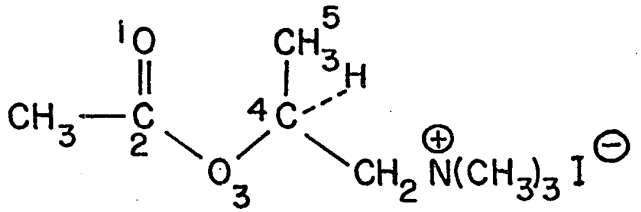
I



XV



IV



XIV

Fig.18 Similarity between the Michaelis complex XV and beta-methyl acetylcholine XIV.

effect might be operative but would not be detectable by the present methodology.

Substitution of the deuterium at the C_2 position of inhibitor III (ref. Fig.17, p.73) gives no significant change in its inhibition constant. This is to be expected since a CD_3 group is very similar in size to a CH_3 group and only changes in Van der Waals interactions can be expected, thus making it unlikely that a secondary isotope effect could be detected.

The Role of the Acetate Methyl Group of Acetylcholine on the Michaelis Complex Formation

The nature and magnitude of the interaction of the acetate methyl group of ACh with AChE could be estimated through the use of a variety of quaternary salts of the 1-3 dioxolane series. A list and the structures of these compounds are given in Fig.17, p.73. A detailed study of the effect of stereochemistry on affinity for AChE was carried out.

Examination of Fig.17 reveals that within that series of inhibitors, optimal activity is associated with the L(+)-absolute configuration and with a cis-relative configuration about the 2,4- positions of the dioxolane ring. The absence of a cis-methyl group in the 2-position is detrimental to activity as can be seen through a comparison of compounds I and III. The conclusion emerges from these studies that the enzyme's active sites display an absolute stereochemical preference for the asymmetric

arrangement shown in IV (Fig.18, p.74). On the basis of these results, an interpretation relating the conformation of enzyme-bound ACh with the structure of IV (Fig.18) may be proposed.

First, it must be noted that the absolute configuration about carbon 4 of IV (Fig.18) is the same as in D-acetyl beta-methylcholine (XIV) (Fig.18, p.74) which in contrast to its L-enantiomorph, is a substrate for AChE. Therefore, the L° isomer of IV must interact with the same active sites normally binding XIV since carbon 5 of IV ought not to interfere also with binding.

Of special significance is the marked stereospecificity also displayed by the enzyme towards carbon 2, a position which has no equivalence in the substrate XIV but which has its equivalence in Wilson's representation[60] of the enzyme-bound substrate. It should be noted that in such a complex XV, the carbonyl carbon of XIV is now tetrahedral and hence must be asymmetric. It follows that the methyl group in complex XV must be accommodated in a stereospecific manner and it seems probable that the orientation of the methyl group in inhibitor IV would reproduce the preferred orientation of the methyl group in the complex XV. It can be readily seen that the inhibitor IV and the postulated complex XV bear a rather intriguing stereochemical relationship which allows the conclusion that IV may induce in the enzyme a conformational change similar to that induced by the substrate and this through

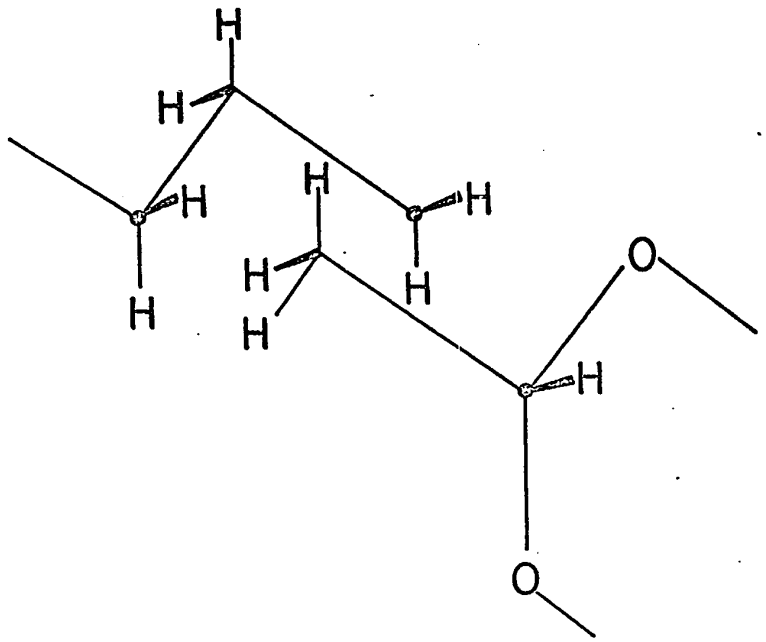
the agency of identical adsorption forces. That this may be so is evidenced by the fact that the affinity of IV for the enzyme is of a magnitude comparable to that of ACh (XVII, Fig.16, p.72). If this interpretation is correct, then it would be the first time that the absolute configuration of a presumed enzyme-bound tetrahedral intermediate can be inferred.

More convincing evidence supporting this theory can be derived from the fact that the role of the methyl group in IV parallels that of the same group in the substrate. Thus formylcholine XVI which is a substrate (analogous to inhibitor I) has a lower affinity for AChE than acetylcholine XVII (Fig.16, p.72) (which has its equivalent in inhibitor IV). It can be seen (Fig.16) that the effect of the methyl group on affinity is the same in the substrate and the corresponding inhibitor series. [It would seem therefore that the methyl group of IV interacts with the same active sites normally involved in the binding of the acetate methyl group of the substrate].

Because no changes in the hybridization of carbon 2 of I and IV occurs in the addition complexes with the enzyme, the absolute contribution of the methyl group of IV to binding can be calculated. The free energy of binding of the C_2-methyl group was found to be -1.35 Kcal/methyl group (ref. Fig.17, p.73). Since the same sites are probably involved in the binding of the same methyl group in the substrate, (the inhibitors being competitive and stereo-

electronically very similar to the substrate) it is likely that this also represents the value for the interaction of the acetate methyl group of ACh with the enzyme. It is of special interest that this value of -1.35 Kcal/methyl group compares quite closely with the value of 1.2 Kcal/methyl group deduced by Wilson for the contribution of each of the first two methyl groups attached to the nitrogen of the substrate or various inhibitors. The nature of the interaction of the N-methyl groups with the enzyme is thought to involve London dispersion forces[60].

A good estimate of the London dispersion forces contributing to the binding of the C_2-methyl group of IV onto the enzyme can be obtained using Salem's treatment[47]. Using two extended chains of methylene groups as a model for the calculation of the London dispersion forces acting across a distance of 5 Å, this author arrived at a value of 0.4 Kcal/mole for the force of interaction between two CH_2 units (ref. Fig.10, p.64). Assuming a hydrophobic region made of the hydrocarbon side chains of various amino acids as being responsible for the imbedding of the methyl group, it should be possible to quantitatively evaluate the magnitude of the forces contributing to such binding. Considering the methyl group of IV as composed of approximately 1.5 CH_2 units enrobed by a chain of three methylene groups equally interacting with the methyl group, (Fig.19, p.79) the total interaction energy can be estimated as 0.6 Kcal/methyl group. This value would



CH_3 GROUP INTERACTING WITH 3 CH_2 UNITS OF LIPID MOLECULE

Fig. 19

account for about half the observed energy of interaction of the C_2 methyl group of ACh with AChE. This apparent discrepancy will be explained later when hydrophobic interactions are considered.

Van der Waals forces are proportional to the polarizability of the bonds in the interacting molecules. Eg. $CHCl_3$ boils higher than methane. This allows the prediction that an increase in the polarizability of the bonds in the 2-methyl substituent of IV (Fig.18, p.74) ought to increase affinity for the enzyme. For instance, changing C-H bonds for C-Cl bonds should lead to stronger London dispersion interactions with non polar side chains since the C-Cl bond is more polarizable than the C-H bond. Examination of Fig.17 reveals in fact, that substitution of the C_2-methyl group of IV by a CCl_3 group as in XII increases affinity for the enzyme by a factor of 4. These results appear to support the above interpretation of the role of London dispersion forces in the binding of the methyl group of the substrate and inhibitors by AChE. (see below for an alternative interpretation).

Hydrophobic Interactions and Affinity for AChE

It was already shown above that Van der Waals forces account for only half of the interaction energy of the C_2 methyl group for the enzyme. In order to account for the other half (0.7 Kcal), solubility studies with the inhibitors were carried out. Solubility data are assembled

Compound No.	Solubility in Water S_W g/l	Sol. in Water S_W m/l	Mole Fraction $N_W \times 10^{-3}$	log Mole Fraction log N_W	Sol. Ethanol S_A g/l	Sol. Ethanol S_A m/l	Mole Fraction $N_A \times 10^{-3}$	log Mole Fraction log N_A	log N_A/N_W
I	482	1.76	30.8	-1.51	23	0.085	4.9	-2.21	-0.70
III	396	1.38	24.2	-1.62	104	0.36	20.6	-1.69	-0.07
VI	463	1.61	28.2	-1.55	112	0.39	22.3	-1.65	-0.10
XI	124	0.41	7.35	-2.13	14	0.046	2.68	-2.57	-0.44
XII	3	0.008	0.13	-3.88	12	0.031	1.75	-2.95	0.93
XIII	14	0.035	0.63	-3.20	47	0.115	6.65	-2.18	1.02
XIX	334	1.18	20.9	-1.68	44	0.14	8.1	-2.10	-0.42

Table 9

Solubility Ratios for the 1-3 Dioxolane Series. (The formulae corresponding to compound numbers are illustrated in Fig.17, p.73).

in Table 9, p.81. The free energy change involved in transferring an inhibitor from an aqueous to a non-aqueous medium (equivalent to the hydrophobic part of the enzyme surface) was determined.(Table 10, p.83). The results indicate a contribution of 800 cal/CH_3 for the transfer of a methyl group from an aqueous to a non-aqueous medium. These results are in close agreement with the results of Cohn and Edsall who used amino acids as models (ref. Table 5, p.45).

An alternative or complementary explanation of the high affinity of inhibitor XII (Fig.17, p.73) for AChE may lie in the high transfer energy associated with the presence of a CCl_3 group (ref. Table 10, p.83). The non-ideality of the solutions obtained with our highly soluble inhibitors (activity coefficients < 1) is undoubtedly a complicating factor in our estimates of transfer energies.

An analysis of these additional results allows us to estimate the relative contribution of hydrophobic forces and Van der Waals attractive energies to the enzyme's affinity for the C_2 substituent in inhibitor IV (see Table 11, p.84). Inhibitor I (Fig.17, p.73) was taken as the standard. The cis-d,l compound III favors both transfer to the enzyme and the operation of positive Van der Waals attractions. The methyl group of its geometrical isomer, the trans-d,l VI, still favors transfer but no longer fits in the same specific way on the enzyme surface.

Of special interest is the effect of two methyl

Table 10

Influence of Structure on Solubility Ratio in Water and Ethanol at 25°C

Substances i and k		$(\log N_A/N_W)_i$	$(\log N_A/N_W)_k$	$\frac{\Delta \log N_A/N_W}{\Delta n}$	ΔF (calculated) Kcalorie
		Influence of CH_3 group			
I	III	-0.70	-0.07	0.63	-0.85
I	VI	-0.70	-0.10	0.60	-0.82
I	XI	-0.70	-0.44	0.26	-0.35
		Influence of CCl_3 group			
I	XII	-0.70	0.93	1.63	-2.28
I	XIII	-0.70	1.02	1.72	-2.34

The formulae corresponding to compound numbers are illustrated in Fig.17, p.73.

Table 11

Relative Contribution of the Transfer (E_t) and Van der Waals (E_v) Attraction Energies to the Enzymic Affinity of the 1-3 Dioxolane Inhibitors C_2 Substituents

Inhibitor	Apparent Interaction Energy		Transfer Energy	Van der Waal Energy
	$+\Delta F_i$	$[(\Delta F_i)_s-(\Delta F_i)]^*$	E_t	E_v
	Kcalorie		Kcalorie	Kcalorie
I	-4.568_s	[0]	0	0
III	-5.923	[-1.355]	-.900	-.600
VI	-4.996	[-.428]	-.400	0
XI	-4.520	[+0.048]	0	0
XII	-6.761	[-2.193]	-2.300	0
XIII	-5.631	[-1.063]	-1100	0

* I was taken as standard

groups at C_2, a situation which is unfavourable to binding. On the basis of the operation of hydrophbbic forces alone, one would have predicted a higher affinity of this compound than with either the cis or trans-monomethyl inhibitors. In fact, its affinity falls short of the expectable value by about one Kcal. An explanation for this discrepancy was found in the abnormally low free energy of transfer of this inhibitor. This must be due to the fact that the methyl groups at C_2 are geminal. Such branching creates an unfavourable energy of interaction with the hydrocarbon portion of the enzyme because a close approach of the inhibitor to this region is forbidden. An interesting illustration of this effect is found in the interactions between isostearic acid molecules. Introduction of branching decreases markedly the attraction energy between the molecules (ref. Introduction, p.45).

Finally, with the CCl_3 compounds the increased affinity can be accounted for solely on the basis of the high transfer energy of a trichloromethyl group (ref. Table 10, p.83). The trans isomer also favors transfer to the hydrophobic region of the enzyme but in this case the resulting interaction would deviate from ideality in a way related to the case of the C_2-dimethyl inhibitor discussed above.

D. Conclusion

The above investigations have brought to light the following new facts concerning the mechanism of complex formation between ACh, related inhibitors and AChE: a) AChE appears to be insensitive to the substitution of deuterium atoms for hydrogens in ACh and in the C_2-methyl group of inhibitor IV. However, the possibility remains that Wilson's representation of the Michaelis complex between ACh and AChE may be adequate; b) The enzyme displays to a high degree absolute and relative stereospecificity towards 1,3-dioxolane inhibitors which bear a close resemblance to ACh itself. Because the mode of interaction between the C_2-methyl group of the inhibitor IV is the same as that between the acetate methyl of ACh and the enzyme, it is concluded that the inhibitor IV reproduces some of the key geometrical features of enzyme-bound ACh. The absolute conformation of the latter can be deduced to be patterned after the geometry of inhibitor IV and it may also be that the uniqueness of the fit produced by this inhibitor is related to a greater tendency for the enzyme to accommodate the tetrahedral arrangement rather than the trigonal geometry for the ACh carbonyl. This would lend some support to the Wilson representation of the Michaelis complex; however, the isotope effect results unfortunately do not allow definite conclusions on this point. c) Variation of affinity constants for the enzyme in the 1,3-dioxolane series can be generally

explained on the basis of variations in the relative contribution of hydrophobic forces to binding. It was shown that the contribution of these forces to binding can serve to explain structure-activity relationships especially when taken in conjunction with the effect of London dispersion forces.

CLAIMS TO ORIGINAL RESEARCH

1. Chemical synthesis of cis 2 methyl D_3-4-trimethylammonium-1,3-dioxolane iodide.

2. Determination of the Michaelis constants (K_m) of four deuterated acetylcholine isomers.

3. Determination of the Michaelis constant (K_m) of formylcholine.

4. Determination of the inhibition constants (K_i) and mode of inhibition (competitive) for the 1,3-dioxolane series.

5. An estimation of the nature and magnitude of the interaction of the acetate methyl group of acetylcholine with acetylcholinesterase.

6. The determination of the role played by hydrophobic forces in the transfer of an inhibitor to the active surface of an enzyme.

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