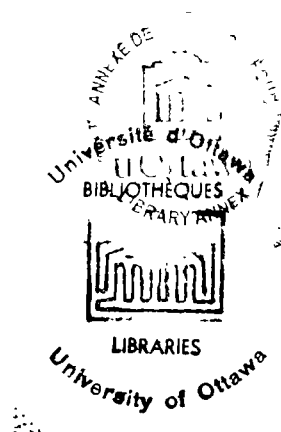


A THERMODYNAMIC STUDY OF THE BINDING OF
COMPETITIVE INHIBITORS TO ACETYLCHOLINESTERASE

by
Jean-Louis Lavoie

A thesis submitted in partial fulfillment of the
requirements for the degree of
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in the
Department of Chemistry
University of Ottawa
Ottawa, Canada



B. Belleau
Professor of Chemistry
Research Supervisor

Jean-Louis Lavoie
Ph.D. Candidate

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FOREWORD

The biochemical importance of acetylcholinesterase¹⁾ arises from the key position that it occupies in the physiology of the nervous system. Following the classical experiments of Loewi in 1921 (1), the chemical theory of nerve impulse transmission at synapses has been proposed (2). According to this theory, the impulse liberates, from the endings of the presynaptic fiber, acetylcholine¹⁾, which then travels to the region of the "receptor" and stimulates it. Following the stimulus, a depolarization of the post-synaptic fiber ensues and the impulse is propagated to the next cell.

As it is released from the receptor site, ACh is hydrolyzed by AChE, which allows for re-polarization to take place again. This step where destruction of ACh is effected is essential to the normal operation of the transmission mechanism, since it ensures the post-synaptic membrane to return to its original state. Would ACh remain intact in the synaptic gap, the post-synaptic fiber would remain in its depolarized state and the propagation of the nerve impulses would be stopped.

Pharmacologists and then kineticists became interested for different reasons in this important neurotransmitter. There resulted a vast quantity of powerful cholinergic and anticholinergic drugs. A great deal of kinetic data and mechanism studies on the hydrolysis of ACh by

1) Abbreviations used: acetylcholinesterase = AChE (EC 3.1.1.7) (22)
and acetylcholine = ACh

AChE have been published with the hope of elucidating the physiological role of this unique enzyme-substrate system.

On the other hand, with the advent of new technical methods of analysis, it now seems possible to integrate kinetic data with the results of studies on the macromolecular properties of the enzyme, thus making it possible to extrapolate in vitro results to the complex situation that prevails in intact preparations. Obviously, for our purposes, a discussion of the mechanism of action of acetylcholinesterase must bear some considerations to its relationships with the post-synaptic receptor.

The possibility that the ACh-receptor may be the enzyme AChE itself, (that is, ACh would be hydrolyzed at the same site at which it stimulates the receptor) is not new. This hypothesis has been advanced a long time ago by Roepke (122) and was revived more recently by Županić (123). Today with more knowledge available on the properties of membranes in general (3) and on the ACh-receptor (4), the problem of the structure and functions of this receptor acquires renewed interest. In the recent literature (80, 115), a very close association tends to be made between certain properties of the enzyme and the possibility that it makes an actual part of the highly elaborate structure which is responsible for the properties of permeable membranes. There is still a great deal of speculation, but this only indicates that advantage may be gained from analyzing certain in vitro results in relation to specific properties of more complex molecular networks.

In this work, the thermodynamic approach has been used to analyze the events occurring at the active site of AChE upon binding of small quaternary molecules which may be substrates or inhibitors. As far as we know, only once before has a fairly comprehensive study of the thermodynamics of binding equilibria been reported as an approach to the problem of interaction mechanisms between small molecules and proteins. This work was carried out with trypsin by T. Inagami in 1963 (124).

The experimental procedure used here is a rather classical one: the catalysis of the hydrolysis of ACh by AChE was used as the method to determine the binding of inhibitors. The results, on the other hand, are discussed in terms of a new physical model as applied to the enzyme itself as well as the corresponding physiological receptor.

This present work represents a contribution to the construction of this physical model which will be referred to as the "water extrusion hypothesis" (5). According to this model, there would exist for all substrates and inhibitors that bind to AChE, a common physical mechanism of interaction that controls the binding and which determines the specificity of the interactions both with the enzyme and the membrane receptor for ACh.

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ABSTRACT

This thesis is divided into five chapters.

In the first chapter, which is the introduction, one finds a fraction of the knowledge on acetylcholinesterase, its kinetic as well as mechanistic properties. We have selected to present only the material which is immediately pertinent to the topic under consideration. In this same chapter, are given the essentials on "the types of forces involved in binding" as well as on "the extrathermodynamic relationships", both being subjects that are referred to implicitly in the discussion.

Chapter II concerns the experimental methods, where details are given about the chemical materials used, the procedure employed for the measurements as well as the numerical and graphical evaluation of the data.

The results are assembled in chapter III, in the form of seven tables illustrated by means of seven different figures. These results are primarily those obtained from the kinetic measurements and secondly those arisen from the statistical treatment applied to the kinetic data.

In chapter IV, these results are discussed. In the whole set of data certain particular trends are discovered that lead to the pro-

posal of a physical interaction mechanism which tries to explain the interaction specificities encountered in the AChE-ligand complex formation. The proposed model is essentially as follows: a ligand which binds onto the AChE surface extrudes from this surface a certain number of water molecules. If, in addition, this ligand is endowed with such structural properties that it will extrude strategically bound water molecules, a perturbation in the protein conformation will ensue, which is an exclusive property of specific ligands, such as ACh.

Chapter V concludes in showing that the proposed model is not necessarily restricted to the case investigated but that it may profitably find application in other systems of biochemical interest.

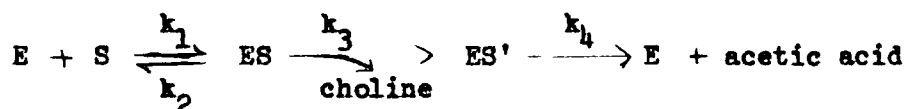
INTRODUCTION

Acetylcholinesterase: An enormous amount of data is available for this enzyme as is the case for many other hydrolytic enzymes. We shall retain only these important data that are pertinent to the work presented here.

source: AChE is found in the nervous tissue of all living matter and its function is to split the acetyl ester of choline (6). The common practical sources for the enzyme are almost exclusively the erythrocytes of beef blood and the electric tissue of Electrophorus electricus, although different tissues of other species are sometimes used, such as brain. The specificity of the cell enzyme (true cholinesterase) is almost absolute, the best substrate being ACh. In contrast, the enzyme isolated from serum (pseudocholinesterase) has a much lower specificity. Throughout this work, and unless otherwise specified, AChE refers to the specific enzyme isolated from beef erythrocytes. As far as the kinetic and molecular weight properties of the enzyme are concerned, no evidence for fundamental differences between the erythrocyte and electric eel enzyme has yet been produced (12, 13, 14). It has been generally accepted (26), that these enzymes from different sources are identical so that all the data presented here could presumably have been secured by working with the electric tissue of E. electricus.

kinetic properties: following the studies of groups of workers like those of Nachmansohn, Bergmann and Wilson, the overall kinetic properties of this enzyme have been largely elucidated and were found to be consistent with the general Michaelis-Menten theory of enzyme catalysis: the catalysis occurs through the formation of an enzyme-substrate (ES) complex which in the subsequent step (acylation) yields an acyl-enzyme complex (ES') and choline (7, 19, 24). The acyl-enzyme then undergoes hydrolysis to acetic acid in the deacylation step which is rate-limiting (for the case where acetylcholine is the substrate) (8) (see scheme I).

Scheme I



where E is AChE, S is ACh, ES is the classical Michaelis complex.

The steady-state treatment of such kinetic events can be found in numerous textbooks which illustrate the classical Michaelis-Menten theory of enzyme mechanisms (13, 41, 42). The rate V_o of a reaction following scheme I is given as

$$V_o = \frac{k_3 [E_o] [S]}{K_m + [S]}$$

where $[E]_0$ is the initial concentration of enzyme
 $[S]$ is the initial concentration of substrate
 K_m the Michaelis constant, equals

$$\left(\frac{k_2 + k_3}{k_1} \right) \left(\frac{k_4}{k_3 + k_4} \right)$$

which reduces to $\left(\frac{k_2 + k_3}{k_1} \right)$ when $k_4 \gg k_3$

V_m the maximum velocity is $k_3 [E]_0$

Two regions characterize that portion of the enzyme surface responsible for the process of hydrolysis. One is responsible for the binding of the ammonium moiety in acetylcholine and known as the anionic site and the other, called the esteratic site is more directly concerned with the hydrolytic reaction (9, 10). The anionic site, through coulombic attractions (11) would be mainly responsible for the total binding of acetylcholine, while the esteratic site would contribute only weakly (12). The nature of the functional groups responsible on the one hand for the binding at the anionic site, and on the other for catalysis at the esteratic site cannot as yet be defined with certainty although many proposals have been made on this subject.

Some evidence would point to the presence at the anionic center of a carboxylate ion presumably supplied by a glutamic acid side chain

(13, 14). For many years, a controversy existed as to whether there are one or two negative charges at the anionic center of true cholinesterase. On the basis of calculated interaction forces between the enzymes and charged substrates and by comparison with uncharged substrates, Bergmann proposed the existence of a doubly-charged anionic center (15, 16) as opposed to a singly-charged center for the case of pseudocholinesterase. On the other hand, I.B. Wilson (114) proposed on essentially similar grounds, the alternative that the center of pseudocholinesterase would bear no negative charge, while only one such charge would be present at the anionic center of true cholinesterase. At the present time, an analysis of the role of Van der Waals forces between AChE and its substrates, has led to the generally accepted view (13) that Wilson's proposal of a singly-charged center may be correct.

The pH-dependent activity of AChE (27) has been interpreted as establishing the presence at the active site and particularly at the esteratic site of dissociable groups participating in the hydrolysis of substrates; both an acidic and a basic group would be present at this site with respective pK values of 7.2 and 9.3. Later, other data obtained with the electric eel enzyme led to estimated pK values of 6.5 and 9.5 (17). The latest studies on the dissociable groups at the esteratic site of true cholinesterase show the possible presence of two basic groups (pK's 6.3 and 5.5) and one acidic group (pK 9.2) with only the group of pK 5.5 being involved and necessary in the formation of the enzyme-substrate-

complex (29). The other basic group of pK 6.3 expected to be situated near the anionic center and the acidic group of pK 9.2 would function exclusively in the acylation and deacylation steps (14). The nature of those functional groups is not known although an imidazole ring is believed to be the group with pK = 6.3.

molecular weight: the impossibility in the past of obtaining AChE in a pure form has hindered progress in the elucidation of its mechanism of action. A very pure preparation has been reported by Wilson (25) who showed it to have an activity of 660 μ moles ACh hydrolyzed per hour per mg. protein. In this case, the source was the electric organ of E. electricus. The enzyme has finally been crystallized recently by W. Lenzinger(138). The activity of the erythrocyte enzyme being highly sensitive to the method of purification, a parallel achievement is yet awaited in this case. The molecular weight of the enzyme has been a source of confusion until recently. Contradictory data have been reported on this problem: for instance molecular weights ranging from a few millions to a few thousands for the unit-polymer have been reported. Recently, a molecular weight of some 230,000 for an aggregate of four subunits has been claimed (23). It is not known whether or not the protomer can still be further dissociated, but Wilson's data (23) are presently considered as reliable, especially since it is now known that the state of aggregation of the molecule depends entirely on the method of isolation (18).

Changeux (80) established, using AChE from Torpedo marmorata, that the state of aggregation is highly dependent on the ionic strength of the medium, with the small enzyme unit (M.W. \sim 250,000) aggregating reversibly at low ionic strength. Similar conclusions are reported in detail by Grafius and Millar (115) who worked with the E. electricus enzymes. The ionic strength and the pH were found to have a profound influence on the homogeneity of the polymeric structures. At the same time, direct relationships were established between the state of aggregation and the kinetic properties of the enzyme. These new findings complicate the overall picture of the structure and mode of action of this enzyme to such a degree that one can no longer ascribe a definite structure to the physiologically active form on the basis of in vitro experimental findings.

It goes without saying that the amino acid sequence of the enzyme is unknown as yet, so that the topography of the active site remains obscure. However, the involvement of an active serine hydroxyl group at the esteratic site has been reported a few years ago by Summerson (20) and confirmed more recently by Sanger (21). The isolation of diisopropyl phosphoryl-acetylcholinesterase made possible to establish the following sequence around the active site: Glu-Ser-Ala, a sequence which is common to AChE from electric tissue, to pseudocholinesterase and horse liver esterase. A more recent interpretation of the chemistry of the diisopropyl phosphoryl-enzyme has confirmed the involvement of a serine residue in the structure of the active site (79).

Forces involved in binding: The interactions between proteins and small molecules are known to involve different types of forces, both attractive and repulsive, the vectorial sum of which will determine the intensity of the interaction. Discussions of these forces as they apply to the interactions between enzymes and substrates or inhibitors can be found in many classical references dealing with this subject, (30,72). The attractive forces may be summarized as follows:

- a) ionic bonds will strongly contribute to the binding of charged ligands as in the case of arginine derivatives which bind on trypsin or of alkyl ammonium ion inhibitors which interact with acetylcholinesterase. The formation of these bonds is initiated by the electrostatic attraction between unlike charges but entropy contributes heavily because of the destruction of the hydration shells around the ions.
- b) hydrophobic bonds, they originate from the tendency for non-polar groups to adhere to one another in an aqueous medium. The transfer of a non-polar group from water to a non-polar environment is mainly entropy driven, since the transfer to the non-aqueous phase is accompanied by the destruction of "iceberg water" surrounding the non-polar group. The application of such forces has been recently demonstrated in studies on the binding of detergent-like molecules to bovine serum albumin (48), β -lactoglobulins (32) and bovine haemoglobin (116). They are rather non-specific and the solubilization of butane and pentane by some enzymes stresses the existence and the key role of hydrophobic crevices on protein

surfaces (33). The contribution of this type of bonding may be as high as 2-5 Kcal/mole as was shown in the case of the inhibition by a series of aromatic compounds of α -chymotrypsin (120).

c) hydrogen-bonding, the secondary structure of proteins depends to a large extent on the presence of intra-molecular H-bonding, especially in the formation of the α -helix configuration in certain regions of the polypeptide chain (121). But only in very few cases, is the operation of this type of binding for small molecules, based on good experimental evidence. The strong inhibition constant for the binding of the 3-hydroxy-phenyl trimethyl ammonium ion to AChE has been explained on the basis of specific H-bonding effects by Wilson (60). The energy of this bond is believed to amount to 3 Kcal/mole, a value calculated on the basis of observed differences with molecules where the formation of this bond would not be possible. But in most other cases, its postulated involvement is usually considered in cases of unusually high affinity constant but without much direct evidence. This type of bond whose energy ranges from 1 to 5 Kcal/mole can in principle contribute largely to the binding of small molecules.

d) dispersion forces, their application to protein interactions has been a subject of controversy. For example, in the binding of charged and uncharged substrates on AChE, there still exists some disagreement as to their net involvement. These forces are known to decrease rapidly with the 7th power of the intermolecular distance, and although they

probably apply in the juxtaposition of like-residues (the interaction hydrocarbon chain - hydrocarbon chain is favored by 1 Kcal over the interaction hydrocarbon chain - aromatic ring), their net involvement in protein - small molecules interactions is not firmly established because of the complexity of the parameters which influence the energetics of the water-macromolecule interactions.

e) the formation of covalent bonds between proteins and small molecules occurs in only a few cases, as for example in the reaction of irreversible inhibitors with active sites and in the formation of acyl-enzyme complexes, which in any event cannot be treated as systems in equilibrium. Their application is restricted to isolated cases.

Interaction with substrates: When are present in a ligand, those features that may favor the application of one or the other of the various types of interaction forces summarized above, binding on the protein will obviously be enhanced. Conversely, preventing the application of these forces should result in a poorer binding. But the evaluation and the actual interpretation of the intensity of a binding force represents a formidable challenge.

Following Scheme I, the Michaelis constant K_m was defined as follows:

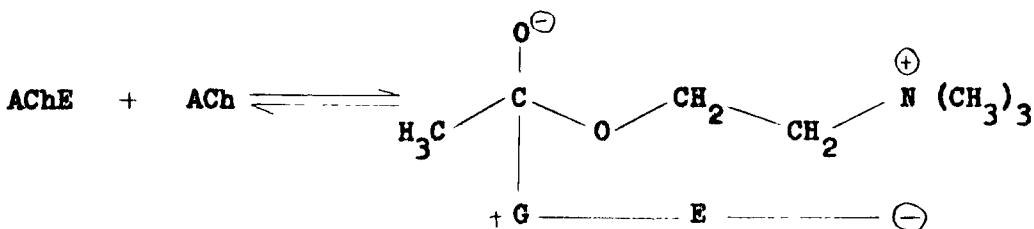
$$K_m = \left(\frac{k_2 + k_3}{k_1} \right) \left(\frac{k_4}{k_3 + k_4} \right) .$$

According to classical kinetic treatments, K_m cannot be defined as an equilibrium constant K_s (the association constant for the formation of ES), unless the relative values of the rate constants are known, which is rarely the case. Generally, K_m is rather considered as a steady-state constant and cannot under those conditions allow an estimate of the actual forces involved in the binding step with the enzyme. Nevertheless, even if it is not possible to directly ascribe the effect of a change of structure on the binding, one can experimentally appreciate using the Michaelis constant, that such or such modification produces a relatively better or poorer substrate.

The problems associated with the formation of an enzyme-substrate complex can be illustrated with the ACh-AChE system. Changes of structure in the substrate modify the Michaelis constant and such changes are currently being analyzed in terms of specific forces of interaction using as a basis the assumption that the binding of ACh occurs through a nucleophilic attack on the carbonyl group with the result that the carbon atom would acquire a tetrahedral configuration in the addition complex (7, 12).

Following this generally accepted model (Scheme II), structural modifications in the substrate molecule can be examined in accordance with

Scheme II



the view that some structural features will favor the formation of the bound form, while others ought to prevent it. For example, by interfering with the close approach of ACh to the enzyme surface, steric hindrance should impair the postulated nucleophilic attack, as it is believed to be the case for choline esters carrying longer ester functions than acetyl since they are all hydrolyzed at much slower rates than ACh itself (12). The introduction in the choline moiety of some substituent susceptible to stereo specifically interfere with the binding, leads to similar observations, as is the case for the α -methyl acetylcholines which are poorer substrates than ACh (34).

By contrast with these types of steric effects, the removal of an N-methyl group in ACh leads to an increase in the Michaelis constant, a result linked to the destruction of the coulombic attraction (if the free base is the species that binds) that is believed to take place between the enzyme and its normal substrate. As a proof to this interpretation, Wilson and Cabib (28) calculated that the ΔH value for the step $AChE + ACh \rightleftharpoons AChE - ACh$ is decreased by some 5-9 Kcal/mole with dimethylaminoethylacetate as the substrate, by comparison with ACh. Most of this energy difference is attributed mainly to the absence of a positive charge in the free base form of dimethylaminoethylacetate, and only partly to the absence of the third methyl group of ACh (35, 36).

Lengthening the distance between the nitrogen atom and the ester oxygen was also found to be unfavorable to binding, since the activity

(given as the rate of hydrolysis) was 7% the activity of ACh, when the ethylene group of ACh was replaced by a propylene chain and as low as 0.56% when a butylene chain was present (118, 119).

Variations in the substitution of the ammonium moiety of the acetylcholine molecule results at best in substrates that are good as ACh itself. Classical interpretations, after assuming the non-involvement of the third methyl group of ACh in binding (35), (the group would stick away from the surface), are aimed at explaining the decrease in the rate of hydrolysis of N-demethylated derivatives on the grounds that a decrease in the dispersion forces between the enzyme and the substrates would occur. The assumption that one of the substituents on the ammonium head projects away from the surface, allows the prediction that steric hindrance will be observed only when two large substituents approach the anionic site (118).

The widely accepted picture for the interaction mechanism of ACh with AChE shows that a close approach is necessary for certain forces to be operative and for complex formation to occur. According to this view, a good substrate is one that can fulfill these conditions. But this model suffers many limitations. The following facts may be cited which can hardly be accommodated by the model: the AChE substrate 3-acetoxyquinuclidinium methyl iodide is characterized by a K_m value of 1.53×10^{-4} M, a value almost identical to the K_m for ACh (37); it has recently been reported that 1-(acetoxyethyl) quinuclidinium iodide is not

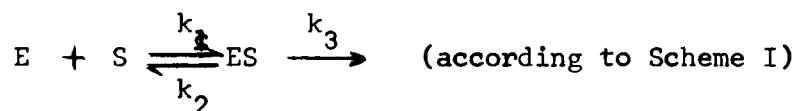
a substrate for erythrocyte AChE but acts as a rather good inhibitor with a K_i (dissociation constant) of 2.2×10^{-4} (38).

It would seem thus that the interpretation of interaction mechanisms and of the substrate specificity of the enzyme on the basis of the above assumed binding forces, leaves unexplained the effects of steric parameters as for example in the case of 1-(acetoxyethyl) quinuclidinium iodide which is not a substrate and 3-(acetoxy)quinuclidinium methyl iodide which is a substrate, even though the same interaction forces should apply to both molecules.

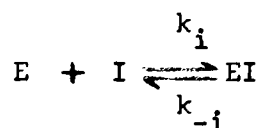
Interaction with inhibitors: The inhibition of catalysis by AChE is the result of the presence on the enzyme surface of a molecule that interferes with the normal process of hydrolysis. The mode of interaction of such molecules forms the basis of the classification into reversible and irreversible inhibitors (39).

The reversible inhibitors form addition complexes with the enzyme that are characterized by a true equilibrium. This can be expressed as in Scheme III for the case of simple competitive inhibition.

Scheme III



$$K_s = \frac{k_2}{k_1}$$



$$K_i = \frac{k_{-i}}{k_i}$$

As can be seen, the enzyme can interact either with the substrate (S) or the inhibitor (I) giving rise to the formation of only two types of complexes ES and EI, where ES is the active form of the enzyme, and EI the inactive form, the relative amounts of which being determined (at a certain enzyme concentration) by the effective concentrations of S and I. The terms K_s and K_i are the respective dissociation constants of ES and EI.

By definition (Scheme I), ES will dissociate by way of one or several kinetic steps to the free enzyme and reaction products. When the formation of EI occurs, a decrease in the free enzyme available for the formation of ES results, and accordingly a decrease in the rate of the reaction will be observed. The steady-state treatment for such a system as depicted in Scheme III leads to an expression for the rate (v_i) of the reaction when pure competitive inhibition takes place (42).

$$v_i = \frac{k_3 [E_0] [S]}{[S] + K_m \left(1 + \frac{[I]}{K_i} \right)}$$

(where the terms keep the same meaning as defined in Scheme I)

The ratio of v_0 (see p. 2) and v_i (above), leads to the following expression

$$\frac{v_0}{v_i} = 1 + \frac{[I]}{K_i \left(1 + \frac{[S]}{K_m} \right)}$$

Thus, if $\frac{v_o}{v_i}$ is plotted against $[I]$, the concentration of the inhibitor, a straight line is obtained, the slope of which is equal to $\frac{1}{K_i \left(1 + \frac{[S]}{K_m} \right)}$ with unity as the intercept when $[I]$ is set equal to zero.

Belonging to the class of pure competitive inhibitors, are the tetraalkyl ammonium compounds and several non-substrate analogs of acetylcholine. As an example, the D-(-)- β -methyl acetylcholine acts as a pure competitive inhibitor of *E. electricus* AChE (34, 43). The alkyltrimethyl ammonium derivatives have also been shown to be competitive inhibitors (83, 95) and generally speaking, derivatives of the tetramethyl ammonium ion series $(CH_3)_3-N^+-CH_2-R$, where R can vary considerably, behave as pure competitive inhibitors of AChE (45). Muscarine, a powerful cholinergic agent has been found to act as a pure competitive inhibitor of electric eel AChE, and is characterized by a K_i dissociation of 2.2×10^{-4} M (46), while the value for choline is 4.5×10^{-4} (13 p.287). In other words, those substances that can bind to the anionic center generally exhibit a pure competitive action.

Many compounds have gained historical importance because of their specific physiological effects on the nervous system. Some of those are currently referred to as the "nerve gases", and their efficacy and toxicity have been attributed to the irreversibility of their action on the AChE of nervous tissue (47, 12). The structure of those so-called "oxydiaphoric" inhibitors is such that the transfer of an acyl-like group

(such as phosphoryl) blocks the active center of the enzyme through the formation of a "nearly" undissociable covalent complex.

The oxydiaphoric compounds may often produce an irreversible acylation, phosphorylation (64, 65), carbamylation (51, 62, 63) or sulfonylation (49) of a nucleophile on the enzyme. The irreversibility of their action explains their high toxicity at very low concentrations (10^{-6} - 10^{-10} M for the carbamates). The binding on the enzyme surface is conducive to the formation of a covalent bond with a serine hydroxyl group situated at the active site or in close proximity to the active site, so that the enzyme can no longer function normally (20, 79, 81).

The complexes formed by these non-competitive irreversible inhibitors and the enzyme can in some cases be dissociated with complete regeneration of the active form of the enzyme. The carbamylation of AChE by eserine or prostigmine for example, has been shown, several years ago to be reversed by hydrolysis with water (52). In much the same way, the sulfonylation of chymotrypsin by phenylmethane sulfonyl fluoride can be reversed by acid hydrolysis (50). But in other cases, the inactivation process results in a non-reactivable form of the enzyme. Thus, the organophosphorous compounds, isopropyl methyl phosphonofluoridate forms an undissociable complex with AChE as the result of the formation of a stable covalent bond, with the concomitant elimination of isopropyl alcohol (53). The reactivation of enzymes inhibited by such organophosphates has been the subject of intensive research in the field of enzyme kinetics (54, 55),

as well as in pharmacology (56, 59). One of the main interests lies in the possibility of accelerating the reactivation of the enzyme with certain classes of compounds such as the hydroxamic acids, which have the ability to dissociate covalent complexes. Aldoximes and ketoximes are especially effective in reactivating AChE. A substance such as 2-pyridine aldoxime methiodide (2-PAM) is 10^6 times more active than hydroxylamine in reactivating phosphorylated AChE (12). The 4-isomer of PAM is also active but interestingly the 3-PAM analog is inactive in causing reactivation of the enzyme. Curiously, the same derivative is effective in reactivating the methane-sulfonyl-enzyme, and it is more active in this regard than the 2- or 4-isomers (39, 57). A similar type of specificity has been also observed in the ketoxime series, but in addition to the positional isomerism, geometrical isomerism is also operative in this case. Thus, the anti-isomer is more active than the syn isomer of the 4-derivative in the phenyl ketoxime series while the reverse is true when the oxime group is in the 2-position (55).

The high activity of those compounds has been explained on the grounds that the approach to the phosphorylated site by the nucleophilic group of the reagent would be facilitated by a proper binding on the enzyme surface at a distance that would allow a readier transfer of the bound group. Along this line, Wilson and others have proposed that the reactivators are held through an ion-ion interaction, involving a negative charge situated on the enzyme surface at a fixed distance from the serine hydroxyl group (79, 54, 58).

This concept of complementariness between a small molecule and a given region of the enzyme has formed the basis of an explanation for the potent competitive inhibition produced by the 3-hydroxyphenyl-trimethyl ammonium ion. When compared with the more common inhibitors, of say the series of trimethyl alkyl ammonium derivatives, where the inhibition remains roughly constant at about 10^{-4} M, this inhibitor has a K_i of 3×10^{-7} , which represents a 120 fold increase in strength of binding (or a difference of 3 Kcal) in favor of the phenyltrimethyl ammonium derivative (60). This large difference was ascribed to the possibility for the inhibitor to form a hydrogen-bond with the surface (as mentioned above), because of the highly favorable position of its hydroxyl group. The 2-hydroxy and 4-hydroxy analogs exhibit a much decreased affinity, the reason being that the hydroxyl groups in these cases would be out of reach of the H-bonding forming site.

Models of interactions between proteins and small molecules:

The few examples of interaction mechanisms given in the above sections show that the interpretation of binding phenomena has been usually interpreted in terms of a complementariness between the enzyme and the small molecule, much along the same line as the template theory first elaborated by Emil Fisher (69). In very many cases, a strong interaction between the partners has been interpreted as resulting from a closeness of fit allowing the binding forces to operate at a maximum,

(34, 60, 66, 68). Specific interactions have been necessarily taken to indicate that strong interactions are operative.

This interpretation of complementariness has also influenced the analysis in structural terms of structure-activity relationships in the field of drug-receptor interactions (61). Striking parallelisms between the interactions of substrates or inhibitors with AChE on one hand and of cholinomimetics or cholinolytics with cholinergic receptors on the other, have sometimes been observed with the result that some workers in this field were led to propose identical structures for AChE and the cholinergic receptor (34).

As a correlate, certain ligands possessing very rigid structures and including appropriate reactive groups were used as "compasses" to locate on the enzyme surface the "anchoring" sites (70). The same approach was applied to drug-receptor interactions (34). Although it is very pertinent to the problem of the mechanism of AChE binding, this picture of protein-ligand interactions, directly implies that an actual relationship between strength of interactions and specificity of interaction with the surface is common to all enzymatic systems and receptors. As such, it constitutes a static model of protein binding sites.

On the other hand, Koshland's induced-fit theory of enzyme mechanisms (71, 72) may be considered as an essential first step in the description of the dynamics of enzyme-small molecule interactions. According to this theory, a native enzyme would possess a rather flexible and adaptable structure allowing the active sites to become uncovered somehow, when the

substrate molecule approaches. Through a "conformational" change a geometrically correct cavity would receive the molecule. In the static model, the binding is analyzed solely in terms of static interaction forces; here it is dependent to a large extent on the inducement of a "certain conformational change" by the approaching molecule.

Recent findings on the flexibility and adaptability of protein molecules in general, support the view of a dynamic model for the enzyme active sites. First, the quaternary structure of enzymes was shown to be highly sensitive to the binding of small molecules; aggregation, heat sensitivity and denaturation of proteins were found in very many cases to be influenced by the presence on their surfaces of inhibitors, substrates or coenzymes (75). Then, the existence of allosteric enzymes (73) and the nature of the mechanisms through which they act suggested a re-examination of the problem of interaction specificities (74). The recently proposed mechanisms of Monod et al. (125), of Atkinson et al. (126) or of Koshland (127), proposed to explain allosteric transitions and cooperative effects in the binding of ligands (observed for example in the binding of oxygen to hemoglobin) rest on the basis that protein molecules would undergo a conformational transition triggered by the binding of small molecules (also called effectors). These effector molecules would control the overall process of catalysis.

Experimental evidence for induced conformational changes has been given recently by Koshland in the case of phosphoglucomutase (129) and

glyceraldehyde-3-phosphate dehydrogenase (130). In the first case, the substrate increases the sensitivity of the enzyme towards sulfhydryl group reagents whereas in the second, the coenzyme DPN induces a change in the environment of a reporter group, when it is bound by the enzyme.

Additional evidence for the possible occurrence of induced conformational changes in proteins was provided by X-ray diffraction studies performed on the reduced* and oxidized* forms of hemoglobin from various species. A careful interpretation of such X-ray diffraction, led to the observation of a difference of some 7\AA in the distance separating the haem groups in the oxidized* and reduced* protein respectively. Hence, a conformational change in the protein structure would be induced by the binding of an oxygen molecule (128). This feature is characteristic of the oxygenation reaction, since it is common to the proteins of various animal species.

In the light of these newer concepts on the properties of proteins, the mechanisms of inhibition of AChE as well as the molecular basis for the pharmacological activity of cholinomimetics on the muscarinic receptor required re-examination (77). A molecular theory aimed at explaining the agonistic and antagonistic properties of some AChE inhibitors has emanated from this laboratory and is based on the concept of ligand-induced perturbation of the conformation of the enzyme molecule, a phenomenon that would also apply to the cholinergic receptor. Through this perturbation, a

* "Oxidized" stands for "oxygenated"

"reduced" stands for "deoxygenated"

specific effect is induced either in the activity of the enzyme or in the physiological receptor (78).

A similar suggestion was later made by Changeux (80) who explains a certain type of antagonism of AChE by leptocurares and pachycurares on the basis of a two-conformations model for the enzyme. The conformations would be in equilibrium with each other and would be stabilized by either one of the two types of drugs. This model is a natural correlate of the more general theory on the mechanism of action of allosteric proteins (74).

Scope of the problem:

If the template model for enzyme-small molecule interactions were correct, the characterization of the interaction in terms of binding strength as derived from binding constants and free energy changes may serve to describe the actual mechanism of binding. In this case, the closeness of fit would be the major parameter. But recent evidence, summarized above, shows that the formation of addition complexes with proteins is not as simple. As pointed out, the binding of even very small molecules may induce considerable rearrangements of the conformation of the large molecule. Accordingly, the free energy quantities for binding cannot account for "all" the events associated with the binding. A change in the conformation of the protein around the active site, such as the breaking of certain bonds may well be accompanied by bond making effects at other points,

so that the overall change induced in the protein cannot be reflected in the observed ΔF quantity. This thermodynamic quantity may well remain insensitive to changes in the protein molecule and its immediate environment.

Other thermodynamic parameters like enthalpy and entropy are more likely to be sensitive to any perturbation or conformational change attending the binding of a ligand. As an example, it is only necessary to consider the allosteric transition induced in NADH dehydrogenase by the binding of the effector AMP. While ΔF binding is -4.4 Kcal/mole, the ΔH binding is $+12$ Kcal/mole and the ΔS binding $+57$ e.u. (131). By comparison with the thermodynamic parameters for binding of TMA to AChE ($\Delta F = -3.6$ Kcal/mole, $\Delta H = -6.6$ Kcal/mole, $\Delta S = -10.1$ e.u.) (83), it is clear that the ΔF quantities are almost identical in the two cases and as such do not reflect the important changes undergone by the proteins upon binding of the ligands. The importance and the magnitude of the perturbations can best be appreciated by taking a look at the enthalpy and entropy terms.

Hence, if a perturbation occurs at the active site of AChE upon binding of small molecules, it is more likely to be detected by evaluating and analyzing the enthalpy and entropy quantities, in addition to measuring the binding constants. The mechanisms of interaction can be evaluated properly in terms of modern concepts of protein and enzyme structure only if these parameters are known.

Advantage was taken of the fact that a large body of kinetic data is available in the case of the AChE-inhibitors system, which facilitates an evaluation of the thermodynamic parameters for competitive binding at equilibrium. In order to simplify the interpretation of results, only simple structures have been used in our studies. In this way, a number of new features affecting the interaction mechanisms may be more easily deduced. This study must then not be looked as a definitive investigation of the mechanisms of the interaction but rather as a novel contribution to the elucidation of the parameters that determine the specificity of the enzyme surface.

Enthalpy-entropy relationship:

The thermodynamic approach to the elucidation of the mechanism of organic reactions consists in the analysis of the effects, on the rates of these reactions, of a change in the nature of the solvent or in the substituents of the species undergoing the reaction. The existence of a simple relationship among the thermodynamic quantities for a given reaction does not necessarily establish a unique and definitive mechanism but rather it demonstrates the operation of a "certain type of mechanism". Because such relationships do not form part of the actual structure of thermodynamics, they are often called extrathermodynamic relationships. A relation of the form $\Delta F' = k \Delta F''$, where $\Delta F'$ and $\Delta F''$ represent the free

energy quantities for two processes in which a molecule undergoes a reaction in two related solvents (like cyclohexane and benzene) is an example of such a relationship. Most of the knowledge on this subject has been reviewed by Grunwald and Leffler (94, 93, 110).

An other simple relationship of that kind relates the enthalpy to the entropy changes and has the form $\Delta H = \beta \Delta S$. It applies when the thermodynamic quantities for the reactions of a series of compounds undergoing the same process are known. If the compounds of that series differ only moderately in structure and if the above relationship holds, an additivity rule may be found to apply to the structural changes and the compounds of the series may be said to react by the same mechanism.

But a certain number of assumptions must first be met, namely that the quantities ΔH and ΔS do not vary with temperature, i.e. $\Delta C_p = 0$, which is usually the case over the temperature range of investigation of organic reactions. In addition, the moderate changes that characterize compounds within the same series usually affect only one region of the molecule, or in other words, they include a moiety which presents constant structural features. It is assumed here that the mode of interaction of this constant moiety is not influenced by the presence of different substituents. The validity of such a statement is of course dependent on the nature of the reaction and the nature of the changes introduced as well as to the sensitivity of the constant structural moiety to substituent changes. Of a similar nature is the assumption that the interactions between the

solvent and the reacting species is the sum of the interactions of the various parts of the molecule (94). In this respect, the contribution of the constant structural moiety within a series of reactants to the free energy of the interaction is expected to cancel out with the result that the changes in the thermodynamic quantities will be characteristic of the solvent-substituent interactions.

Under these conditions, and if many pairs of ΔH , ΔS values are known, the relationship between ΔH and ΔS can be shown to apply, if the various compounds of the series react by the same mechanism

$$\Delta H = \beta \Delta S \quad (1)$$

A graph where ΔH is plotted against ΔS will give a straight line of slope β , which has the dimensions of absolute degrees. When $\beta = 0$, the reaction is exclusively entropy driven, but when $1/\beta = 0$ the thermodynamic change is of purely enthalpic origin. The substitution of (1) in the standard equation for ΔF , $\Delta F = \Delta H - T\Delta S$, gives rise to (2) and (3).

$$\Delta F = (\beta - T) \Delta S \quad (2)$$

$$\Delta F = (1 - T/\beta) \Delta H \quad (3)$$

From equations (2) and (3), a special value of β can be defined for which ΔF will be constant in all cases; that is $\beta = T$ ($T = T_{\text{exp.}}$, the mid-point of the absolute temperature range in which the experiments are carried out). This possibility that β be such that ΔF remains constant suggested the expression "isoequilibrium relationship" for equation (1); this special value of β is called the isoequilibrium temperature.

Thus, a constancy in the free energy changes undergone in the reaction of members of a same series does not indicate that the modifications in the structure of the reacting species or in the reaction medium has no effect on the course of the reaction. On the contrary, equations (2) and (3) show that β may have a value equal to or close to $T_{\text{exp.}}$, with the result that the free energy quantities will be nearly constant owing to a mutual cancellation of the terms ΔH and ΔS .

As stated above, the additivity rule represented by the linear relationship (1) indicates the operation of a single mechanism of interaction which is quantitatively modified by the changes introduced by the various substituents or in the solvent; such a mechanism is characterized by a definite value of β . Provided good linearity is observed and if β is very much different from $T_{\text{exp.}}$, it can be concluded then that a single mechanism of interaction is operative. However, in the special case where $\beta = T_{\text{exp.}}$, a good linearity is not a sufficient condition for the establishment of an isoequilibrium relationship. It may happen, as was seen above, that the constancy in rates be due to an insensitivity of

the reaction or the solvent to the structural modifications performed on the substituents. In that case, the correlation coefficient will be close to 1, since constancy in ΔH and ΔS will also be observed.

That is why, in addition to a good linearity, an isoequilibrium relationship with a β value equal to or close to T_{exp} must cover a large spread in both the ΔH and ΔS quantities and this spread must be much larger than the probable errors in these parameters. Only if this condition is respected, can the constancy in rates be ascribed to cancellation effects between the significant fluctuations in ΔH and ΔS (93). When these conditions of linearity over a large range of values for the thermodynamic parameters, are met, a sequence of points whose positions are consistent with the chemical and physical properties of the individuals of the series, also constitutes a test for the veracity of the relationship.

In the list of the many examples of such cases (94) taken from the field of organic chemistry, it is generally seen that a variation in ΔH larger than 3 Kcal/mole is a mechanistically significant one. Moreover, the same literature data reveal that β and T_{exp} can be considered as identical for all practical purposes if the two values do not differ by more than 20° (93).

The physical meaning of a β value which is close to T_{exp} is not very clearly understood, although such a phenomenon is often observed in the field of isoequilibrium or isokinetic relationships. Nevertheless, a β value between 300° and 400° is generally interpreted as indicating that

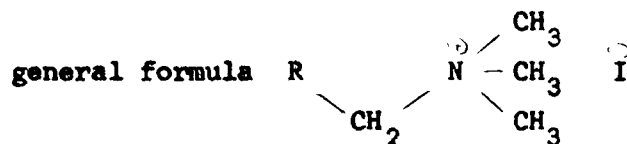
the inflections in ΔH and ΔS are controlled by interactions with the solvent (94). A general analysis of the problem of solvation of reacting species shows that the equilibrium is characterized by minimal changes in ΔF while ΔH and ΔS undergo important variations. In these cases, β is equal to T_{exp} . (111). The dissolution of a series of n-alkanes and n-alkanols in water obeys the isoequilibrium relationship with $\beta = 280^\circ$ and $T_{\text{exp}} = 298^\circ$ (94, 110).

The rates of a certain radical decomposition have been found to be insensitive to the nature of the solvent (112). But evidence for strong interactions between the reacting species and the solvent was obtained from the observation of important fluctuations in the enthalpies and entropies of activation. Thus the insensitivity of the rates is due to large compensation effects in the thermodynamic parameters. In this very case, an isokinetic plot is reported that has a β value of 313° , while the mean value of T_{exp} is 323° .

Extrathermodynamic treatment applied to a series of TMA derivatives (Table I)

The series of the compounds listed in Table I, fulfills the requirements enumerated above for fruitful mechanism studies and the extrathermodynamic approach can then be applied to this series, in so far as the structural modifications are concerned. The changes introduced in the TMA-substituents are moderate, in the sense that the members differ

TABLE I
LIST OF INHIBITORS AND SUBSTRATES



COMPOUND N ^o	NAME a)	MOLAR ^{e)} VOLUME (cc)	MELTING POINTS and Lit. ref.
1	cyclobutyl TMA	~162	200°-200.5° [205.6°-206.5°(101)]
2	cyclopentyl TMA	175.5 ^{d)}	203° [200°-202°(102)]
3	cyclohexyl TMA	190.1 ^{d)}	232°-233° [230°-231°(103)]
4	cycloheptyl TMA	203.2 ^{d)}	237°-238° [235.7°(104)]
5	cyclo octyl TMA	216.7 ^{d)}	265° [265.6°-267.3°(105)]
6	bicyclo [2.2.1] heptyl TMA	~209	282.5°-283.5°
7	bicyclo [2.2.2] octyl TMA	~226	288.5°
8	tricyclo [3.3.1.1.] decyl TMA (adamantyl)	~242	283°-286° (decomp.)
9	2-furyl TMA	~155	115°-116° [116.5°-118.0°(106)]
10	5-methyl-2-furyl TMA	~172	160.5°-161.5° [160°-162°(107)]
11	2-thiophenyl TMA	~162	154.5°-155.5° [152.6°-153°(108)]
12	phenyl TMA b)	170.1 ^{d)}	179.5°-181.5° [178°-180°(109)]
13	L(+)-4-TMA-1,3-dioxolane	~152	148°-150°
14	D(-)-4-TMA-1,3-dioxolane	~152	149°-150°

Table I continued

15	L-cis-4-TMA-2-methyl-1,3-dioxolane	~172	153°-156°
16	L-trans-4-TMA-2-methyl-1,3-dioxolane	~172	124°-128°
17	D-cis-4-TMA-2-methyl-1,3-dioxolane	~172	153°-156°
18	D-trans-4-TMA-2-methyl-1,3-dioxolane	~172	126°-128°
19	2-TMA-1,3-dioxolane (isodioxolane)	~152	197°-199°
20	L(+)-β-methyl acetyl choline	~180	177.5°-179.5°
21	D(-)-β-methyl acetyl choline	~180	178°-179°
22	β-trimethyl ammonium methyl propionate (reversed ACh)	~162	188°-190° [194°-195°(132)]
23	acetyl choline (ACh) ^{b)}	~162	144°-145.5°
24	formyl choline		124.5°-125.5°
25	n-hexyl trimethyl ammonium	212.8 ^{d)}	
26	n-undecyl trimethyl ammonium		

a) TMA stands for tetramethylammonium

b) isolated as its bromide salt

d) data taken from McAuliffe (ref. 96)

e) the volume includes 82.1 ml for the TMA moiety

either by a small change in molar volume (such as the presence or absence of a methyl group) or in the configuration about an asymmetric center. While there exists a constant moiety (TMA²) for all the members, no highly reactive functional groups are found in the substituents as would be the case if a cyano or a hydroxyl group susceptible to introducing undesirable physico-chemical parameters were present. Additivity of the partial molar properties of the ligand substituents must initially be preserved as much as possible.

On the basis of such changes, it is reasonable to postulate that the fundamental interaction of the TMA moiety with the enzyme will be a strict additive function of the cationic head and its substituents. Both the electronic structure of the tetramethyl ammonium and of the substituent are expected not to chemically influence each other. This statement is valid to a first approximation only. But, in this work and for the purpose of studying the effect of structure on the thermodynamic properties of the binding, this assumption is quite reasonable and satisfactory for our purposes.

A similar reasoning applied to the interaction between the various members of the series and the solvent, water in this case. The substituents possess structures different enough so that the hydration layers around them may vary largely within the series. But the changes

² abbreviation used: TMA = tetramethyl ammonium $[-CH_2-N^+(CH_3)_3]$

should be moderate enough so that to a first approximation, the interaction of the TMA moieties with water will be nearly the same for all inhibitors, since little vicinal interactions with the substituents would be expected normally. This second statement is in a sense a correlate of the first one.

It should then be possible to ascribe the effect of the substituent structure on any mechanism that may be described by an extra-thermodynamic relationship, to the nature of the substituent itself, while neglecting any vicinal interactions with the TMA heads. It certainly seems reasonable, to a first approximation, to postulate that all the TMA heads will interact by the same mechanism. Any large differences between the thermodynamic parameters for any two members of the series should be largely relevant to the effect of the substituent alone on the interaction mechanism.

CHAPTER II

EXPERIMENTAL METHODS

A) Materials:

Doubly distilled water (using a glass apparatus) was the only solvent used for all the measurements, as well as for rinsing the electrodes and the cells.

The sodium chloride and magnesium chloride used for the preparation of the isotonic solution were reagent grade chemicals as supplied by Fisher Scientific Company.

Solutions of sodium hydroxide 0.020 N were obtained from Anachemia Chemical Company and the acetyl choline from Matheson Coleman and Bell; the latter was recrystallized three times from absolute ethanol and dried before use.

Preparations of true cholinesterase isolated from bovine erythrocytes and containing sodium chloride and gelatin as stabilizers, were purchased from Nutritional Biochemicals Company.

B) Procedure

The determination of inhibition constants was conducted at four, sometimes five, different temperatures. A thermostated bath was used in order to maintain the temperature constant to within $\pm 0.05^{\circ}$.

The inhibitor and the enzyme were incubated for three minutes before the hydrolysis of ACh was initiated, after which time the temperature of the solution had equilibrated to the desired value. The reaction was started by the rapid injection of substrate.

The kinetic measurements of the hydrolysis of ACh by AChE were carried out using a pH-stat TTT₁ titrator (Radiometer, Copenhagen), to which was connected a G 202 c glass electrode and a calomel electrode of the porous pin type. The pH-stat was also equipped with an adjustable temperature compensator. The titrant, 0.020 N sodium hydroxide was added through a 1 cc. B-D tuberculin syringe driven by the micrometer screw of an Ole Dich recorder. The sodium hydroxide was delivered to the vessel through a 4" special needle # N-728 with an internal diameter of 0.15 mm as obtained from the Hamilton Company Incorporated. It was found that this needle diameter eliminated diffusion of the titrant into the solution.

All reactions were carried out in glass cells, so designed that they would fit into a thermostated jacket. Solutions of the reaction mixtures had a total volume of 25 ml and were prepared as follows: the combined volumes of isotonic and inhibitor solutions amounted to 24.7 ml, to which was added 200 μ l of the enzyme solution and 100 μ l of the substrate solution.

The isotonic solution was 0.1 M in NaCl and 0.04 M in MgCl₂. From a stock solution of 20,000 units of enzyme in 5 ml water, a solution

containing 50 units/ml was prepared, and a total of 10 units were used in each run. (One unit is the amount of activity in 1 ml of the enzyme solution that will catalyze the hydrolysis of one micromole of acetylcholine per minute at pH 7.4 and at 25°C). The enzyme solution was kept at 0°C during the course of the measurements, while the stock solution was kept frozen. Fresh solutions of enzyme and acetylcholine were prepared every second day, despite the fact that acetylcholinesterase is known to retain full activity when kept at 0°C, and that spontaneous hydrolysis of acetylcholine during that period of time is negligible.

Before the reaction was initiated, the pH of the mixture was adjusted to pH 7.4, using 1N sodium hydroxide, so that the increase in volume by this pH adjustment was negligible. During the titration, the pH was kept constant automatically at $\text{pH } 7.4 \pm 0.02$ by the titrator.

Interference by atmospheric carbon dioxide was prevented by passing a slow stream of nitrogen, presaturated with water, over the solution.

The rates of hydrolysis were obtained from the slopes of the curves which give a direct measure of the amount of acid produced per unit time. The curves were linear in every case, so that extrapolations of the recorded lines were permissible as this allowed a more precise evaluation of the slopes. The speed of rotation of the recorder drum could be adjusted at will using different sets of gears, so that the slopes of the curves could be maintained between the interval of 0.8 and 1.6,

a range that minimizes the errors in the graphical evaluation of the slopes.

Calibration of the syringes with respect to the volume of titrant delivered, allowed the slopes to be read directly as fractions of hydrolysis per unit time.

The initial velocities obtained in this manner were calculated for the first 8% of hydrolysis.

In relation with the temperature studies, the fundamental assumption is made that full activity of the enzyme is preserved at all the temperatures used. In other words, it was expected that no denaturation took place under the experimental conditions described.

C) Synthetic procedures:

All the compounds used in these inhibition studies (see Table I) were described previously in literature, with the exception of certain inhibitors of the 1,3-dioxolane series. The synthesis of the compounds required only a few conventional steps involving only well-known procedures. Characterization was accomplished by spectrophotometric methods as well as melting points, which were compared with those reported in literature.

a) cycloalkyltrimethyl ammonium iodides:

Compounds 1-8, compounds 12, 25 and 26 were synthesized following

the method of Cope and Ciganek (84).

A typical experimental procedure as applied to the cycloheptyl derivative is given below; all intermediates were characterized by N.M.R. and I.R. spectroscopy: 25 g. of cycloheptyl carboxylic acid was heated under reflux overnight with 22 g. (5% excess) of thionyl chloride in ether. Then, an ethereal solution of the acid chloride was thoroughly mixed at 0°C with 150 ml of 25% aqueous dimethylamine. Sixteen grams (16 g.) of the amide was thus obtained (B.P. 84°/0.03 mm). The amide was reduced by reaction with a stoichiometric amount of lithium aluminum hydride in boiling ether. After work up in the usual manner, the amine was directly quaternized by the addition of a small excess of methyl iodide in ether. The salt was recrystallized 4-5 times from absolute ethanol. A yield of 13.6 g. of the methiodide (M.P. 237°-238°) was obtained.

b) furane derivatives:

The compounds N° 9 and N° 10 (Table I) were synthesized according to the method described by Ing and Williams (85).

c) 2-trimethyl ammonium methyl-thiophene:

Thiophene was reacted with N-methyl formanilide and phosphorous oxychloride according to the method of Weston and Michaels (86). A quantity of 53 g. of the fraction boiling at 54° under 2.5 mm was collected.

The yield of 2-thiophene carboxaldehyde was 43%. Reduction of the aldehyde (22.4 g.) with lithium aluminum hydride (7.7 g.) gave 15.4 g. of the alcohol. The latter was not purified but directly reacted with thionyl chloride to give the 2-chloromethyl thiophene derivative. The yield of fraction boiling at 42°/0.60 mm was quantitative. An ethereal solution of the chloride was added to a solution of dimethylamine in tetrahydrofuran and stirring was continued overnight. The product was extracted, washed and dried. Then the methiodide salt was formed by reaction of the amine with an excess of methyl iodide in ether at 0°C overnight. After three recrystallizations from absolute ethanol, 7.0 g. of the salt (M.P. 154.5°-155.5°) was isolated.

d) 1,3-dioxolane derivatives:

The experimental procedure for the stereospecific synthesis of these inhibitors were described previously (87, 88). The same techniques were applied here, with the exception that the recovery of the trans diastereo isomers from the mother liquors was accomplished using chromatographic procedures to be described by Dr. V. DiTullio and Dr. D. Wiewioroska.

e) D-(-) and L-(+)-β-methyl acetylcholine iodides:

The synthesis of these compounds (N° 20 and N° 21) was accomplished by the method already reported by A.H. Beckett (89) with slight

modifications. The resolution of N,N-dimethyl amino isopropanol was carried out using (-) and (+) tartaric acids for the respective isolation of the L(+) and D(-) enantiomers. The tartrates were recrystallized from 95% ethanol until the salts had the reported optical rotation. Unlike the recommended directions which call for 3 recrystallizations, some twelve recrystallizations were found to be necessary in order to achieve optical purity. The optical activity of (-)- β -dimethyl amino isopropanol acid-(+)-tartrate was -10.0° (c.5% in water) as measured with a Rudolf polarimeter model 62. In the case of the (+)- β -dimethyl amino isopropanol acid (-) tartrate, the α_D was 9.0° (c.5% in water) and remained constant after three additional recrystallizations. The optical activities of the final products were as follows:

$$\alpha_D \text{ D(-)-}\beta\text{-methyl acetyl choline iodide} = -26.5^{\circ\text{b}}$$

(c.2.0 in 95% ethanol)

$$\alpha_D \text{ L(+)-}\beta\text{-methyl acetyl choline iodide} = +28.7^{\circ\text{a}}$$

(c.2.0 in 95% ethanol)

The reported α_D values as given by Beckett are:

for the L(+) isomer	$\alpha = +27^{\circ}$	(c.2.0 in 96% ethanol)
D(-) isomer	$\alpha = -25.7^{\circ}$	(c.2.0 in 96% ethanol)

a) measured on a Rudolf polarimeter model 62

b) measured on a Perkin-Elmer polarimeter model 141

f) β -trimethyl ammonium methyl propionate iodide:

The synthesis of this compound as described by W.B. Bass et al (90) involves the condensation of methyl 3-bromo propionate with trimethyl amine in benzene.

g) formyl choline iodide:

A standard method of preparation was used that involves the condensation of 2-iodoethyl formate with trimethyl amine in ether at room temperature. The iodoethyl formate was prepared by heating under reflux a benzene solution of anhydrous formic acid and 2-iodoethanol, using a Dean-Stark trap to remove the water. The purity of the compound was ascertained by I.R. and N.M.R. spectroscopy. A strong band at 1710 cm^{-1} which is characteristic of the ester carbonyl was observed.

D) Graphical Determinations:

a) Inhibition constants:

As was discussed in Chapter I, plots of V_0/V_i versus the concentrations of inhibitor allows the value of the inhibition constant K_i to be calculated from the slope in the case of competitive inhibition. In these determinations, enough inhibitor was used in order that V_0/V_i varies by a factor as large as 3 or 4. The velocities of the inhibited reactions were evaluated at a minimum of four different inhibitor

concentrations. Duplicate and sometimes triplicate measurements were always carried out for each inhibitor. Upper and lower limits for the slopes (and for K_1 's) were thus calculated. The magnitudes of the maximum deviations are illustrated on the Arrhenius plots of Figure 5.

b) Michaelis constant for ACh:

The mean value of K_m for ACh was obtained at 25°C and found to be $3.25 \pm 0.15 \times 10^{-4}$ M. It was evaluated by a least square analysis of the Eadie and Lineweaver-Burk graphical representations. This value is in agreement with previous determinations: 3.8×10^{-4} M (44), 4.5×10^{-4} M (45), 5×10^{-4} M in presence of 0.2 M $MgCl_2$ (80), 2.1×10^{-4} M (37) and 3.3×10^{-4} M with the housefly head enzyme (117).

The concentration of ACh in the reaction medium was 6.5×10^{-4} M in all cases.

As previously established by Wilson (28) and Shukuya (82), and confirmed more recently by Belleau et al (45, 83), the value of K_m (app.) for ACh was found to be constant at all the temperatures studied. Therefore, the value of 3.25×10^{-4} M determined at 25°C was used in all calculations of inhibition constants, in the temperature range of 10°-30°C.

c) Evaluation of enthalpies of binding:

The K_1 being a true equilibrium constant, as defined above (see Scheme III), the following relationship for its temperature-dependence is applicable (133):

$$\frac{d(\ln K_1)}{d(1/T)} = - \frac{\Delta H}{R}$$

A graph where $\ln K_1$ is plotted against the reciprocal of the absolute temperature gives a straight line where the slope is equal to $-\Delta H/R$, R being equal to 1.98 cal. deg.⁻¹ mole⁻¹.

Average values of two and often three determinations of K_1 at each temperature were plotted against $1/T$ and the slopes of the curves measured in order to calculate the corresponding ΔH values. The deviations in ΔH , as expressed in Table IV, originate from the experimental error in the determination of K_1 's and were obtained graphically. In the range of temperature studied, the Arrhenius plots were always linear.

E) Calculations:

The free energy changes (ΔF binding) were calculated using the expression

$$\Delta F = -2.303 RT \log K_1$$

where K_1 is the binding constant at equilibrium, T is the temperature in absolute degrees and R is the gas constant (1.98 cal. deg.⁻¹ mole⁻¹). The deviations in the evaluation of ΔF , were related to the experimental error in the determination of K_1 's.

In the case of acetylcholine and β -methyl acetylcholine, apparent Michaelis constants are determined. Without any consideration being given as to whether these constants are true equilibrium constants,

the $K_{m\text{app}}$ values have been treated as if they had the same meaning as the K_i 's and accordingly, the apparent thermodynamic quantities are given by the following relationships:

$$\Delta F_{\text{app.}} = -RT \ln K_m$$

$$\Delta H_{\text{app.}} = -R \frac{d(\ln K_m)}{d(1/T)}$$

$$\Delta S_{\text{app.}} = \frac{\Delta H_{\text{app.}} - \Delta F_{\text{app.}}}{T}$$

These equations were applied without any theoretical considerations, a procedure which was similarly followed in the case of α -amylase (113); the purpose here is to compare the thermodynamic quantities for substrates and other ligands.

The entropy change upon binding was calculated according to the classical equation

$$\Delta S = \frac{\Delta H - \Delta F}{T}$$

in which the measured ΔF and ΔH values were inserted.

The errors found on this quantity were the sum of the errors on the experimental determinations of ΔF and ΔH .

CHAPTER III

RESULTS

1) Inhibition Constants:

Inhibition constants (dissociation constants of the enzyme-inhibitor complexes) at different temperatures for compounds 1 to 19, 21, 22, 25, and 26, (Table I) were determined and are listed in Table II. Typical plots of V_0/V_i vs inhibitor concentration are shown in Figures 1, 2, and 3, on pages 48, 49, 50; these illustrations are representative of each class of chemical structures. These graphs also illustrate the competitive character of all those inhibitors. The competitive behavior was observed in all cases and at all temperatures, thus confirming the previous results of Belleau and Lacasse (45) for the dioxolane series and of Belleau et al (83) for the alkyl trimethyl ammonium series.

2) Compounds with an Ester Function:

a) D(-)- β -methyl acetylcholine (compound N^o 21) and reversed ACh (compound N^o 22):

Those two substances were found not to hydrolyze spontaneously in water, at least during the time of the experiments. Neither did they act as substrates for AChE.

TABLE II
 Variation of $K_i^{b)}$ with temperature
for derivatives of tetramethylammonium (TMA)

Compound N ^o	TMA Derivative	$K_i \times 10^4$				
		10 ^o	15 ^o	20 ^o	25 ^o	30 ^o
1	cyclobutyl	2.39		2.93	3.12	3.40
2	cyclopentyl	2.54	2.75	3.10		3.60
3	cyclohexyl	6.26		6.62	6.60	6.71
4	cycloheptyl	4.68	5.38	6.37	6.94	8.03
5	cyclo octyl		4.08	4.94	5.38	6.49
6	bicycloheptyl		9.71	8.85	9.52	9.13
7	bicyclo octyl	9.66		9.48	9.50	9.48
8	tricyclodecyl		6.21	7.17	7.46	8.23
9	furyl	2.94	2.98	3.00	2.91	3.07
10	methylfuryl	4.67	5.05	4.99	5.25	5.33
11	thiophenyl	2.20	2.56	2.93	3.53	4.13

Table II continued

12	phenyl		4.17	4.22	4.17	4.37
13	4-L-(+)-1,3-dioxolane	6.21		8.97	10.52	12.91
14	4-D-(-)-1,3-dioxolane	13.26		15.55	16.80	18.28
15	4-L-cis-2-methyl-1,3-dioxolane	4.00		4.26	4.68	4.72
16	4-L-trans-2-methyl-1,3-dioxolane	13.33		15.55	17.03	17.48
17	4-D-cis-2-methyl-1,3-dioxolane	11.33		13.33	14.66	16.18
18	4-D-trans-2-methyl-1,3-dioxolane	13.42		16.10	17.12	18.69
19	2-1,3-dioxolane (isodioxolane)	6.19		6.73		7.49
20	L(+)- β -methylacetylcholine ^{ac)}	18.7	17.7	16.5	15.5	14.4
21	D(-)- β -methylacetylcholine ^{a)}	15.00		19.72	23.31	25.31
22	β -trimethylammonium methyl propionate ^{ab)} (reversed ACh)	24.50	29.94	31.25		41.66
23	acetylcholine (ACh) ^{a)}				3.25	
25(C ₆)	n-hexyl	7.58		9.86	10.24	12.28
26(C ₁₁)	n-undecyl	0.65		0.72	0.81	0.85

a) Full name is given

b) K_1 is a dissociation constant

c) Variation with temperature of apparent Michaelis constants

Figure 1

Variation with temperature of the inhibition of ACh
hydrolysis by AChE for cyclo butyltrimethylammonium
iodide (compound N° 1) at four temperatures

The inhibition is of the pure competitive type.

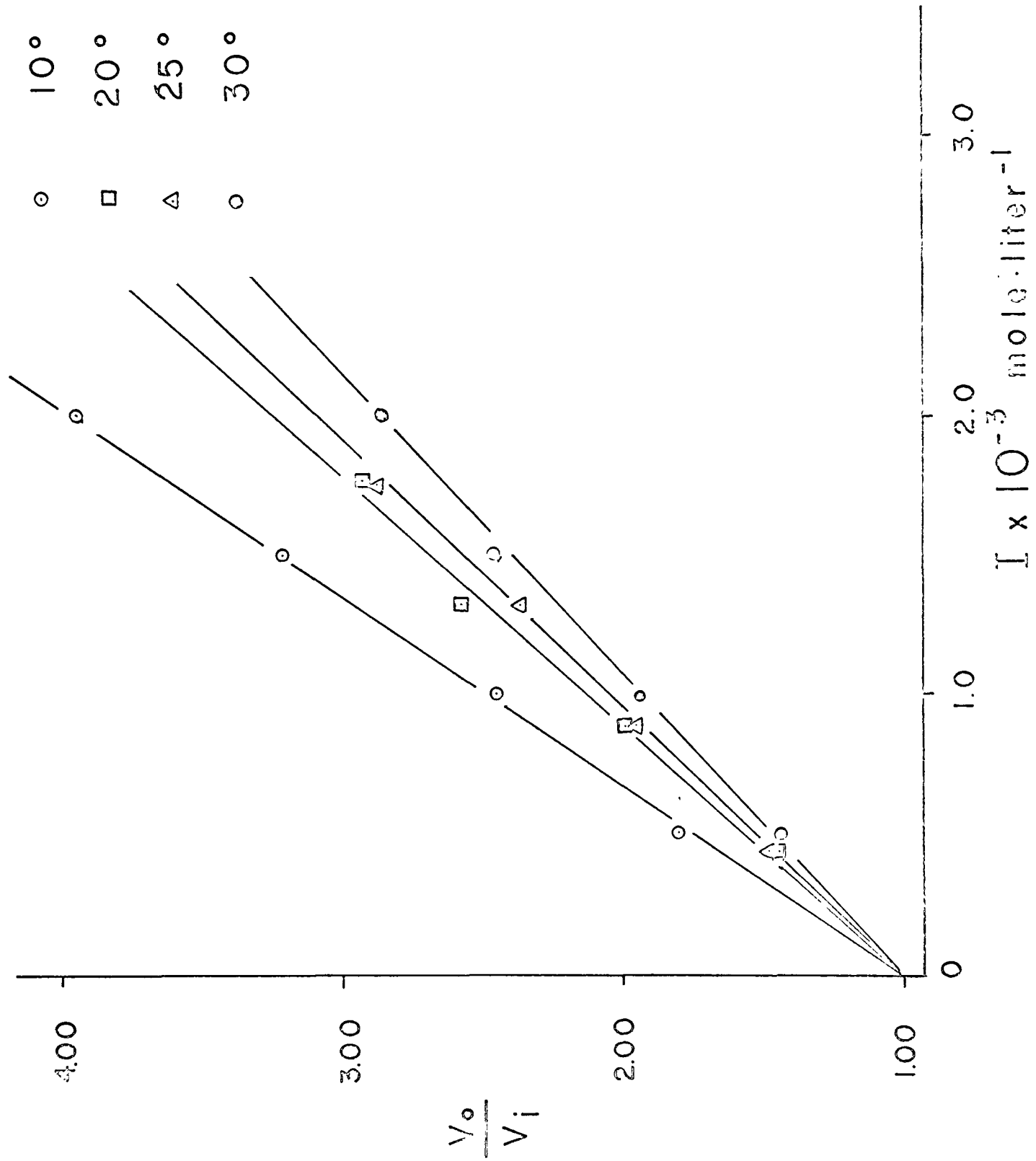


Figure 2

Variation with temperature of the inhibition of ACh
hydrolysis by AChE for the cyclo octyl trimethyl ammonium
iodide (compound N° 5), at three different temperatures

The inhibition is of the pure competitive type

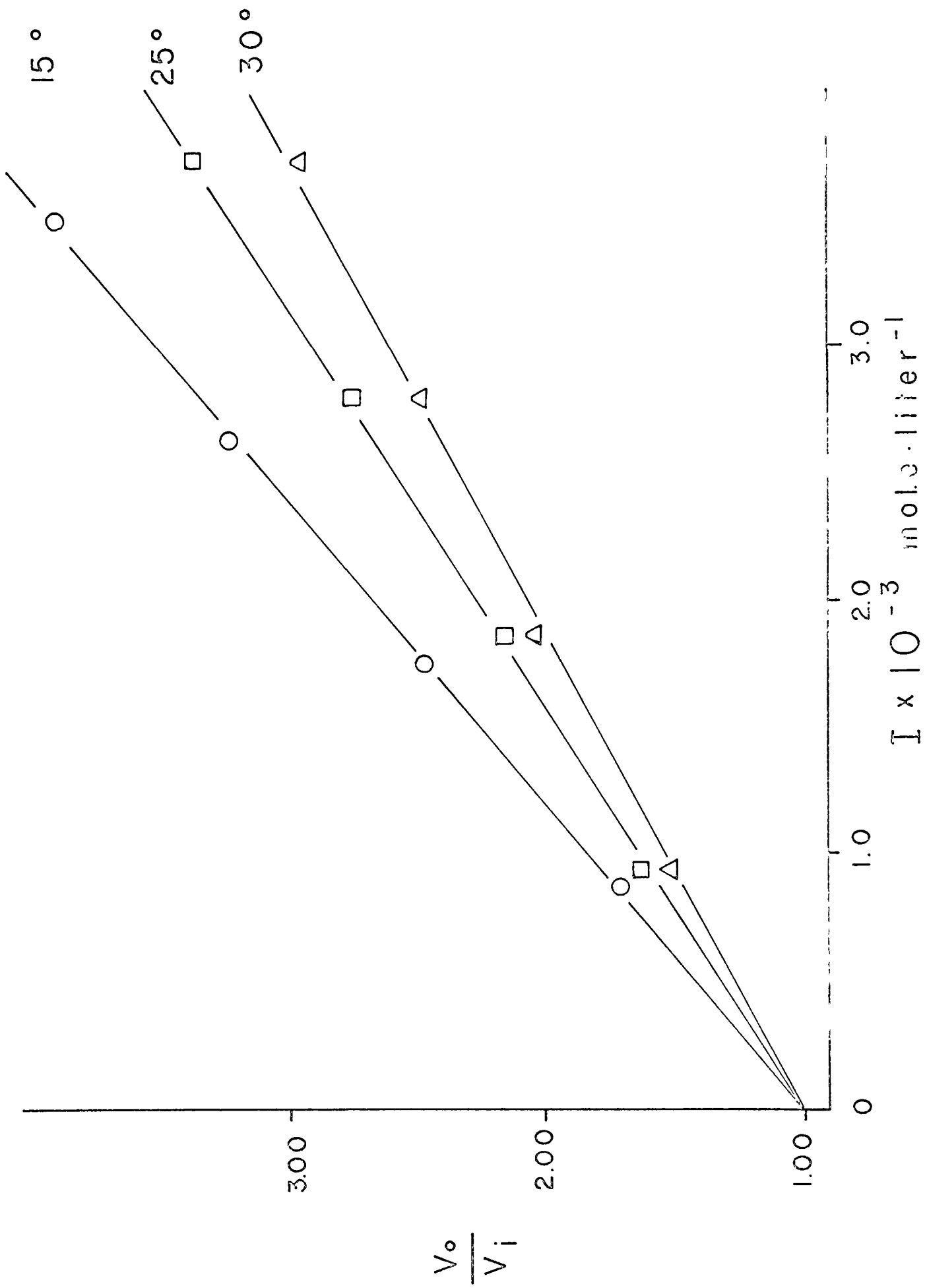


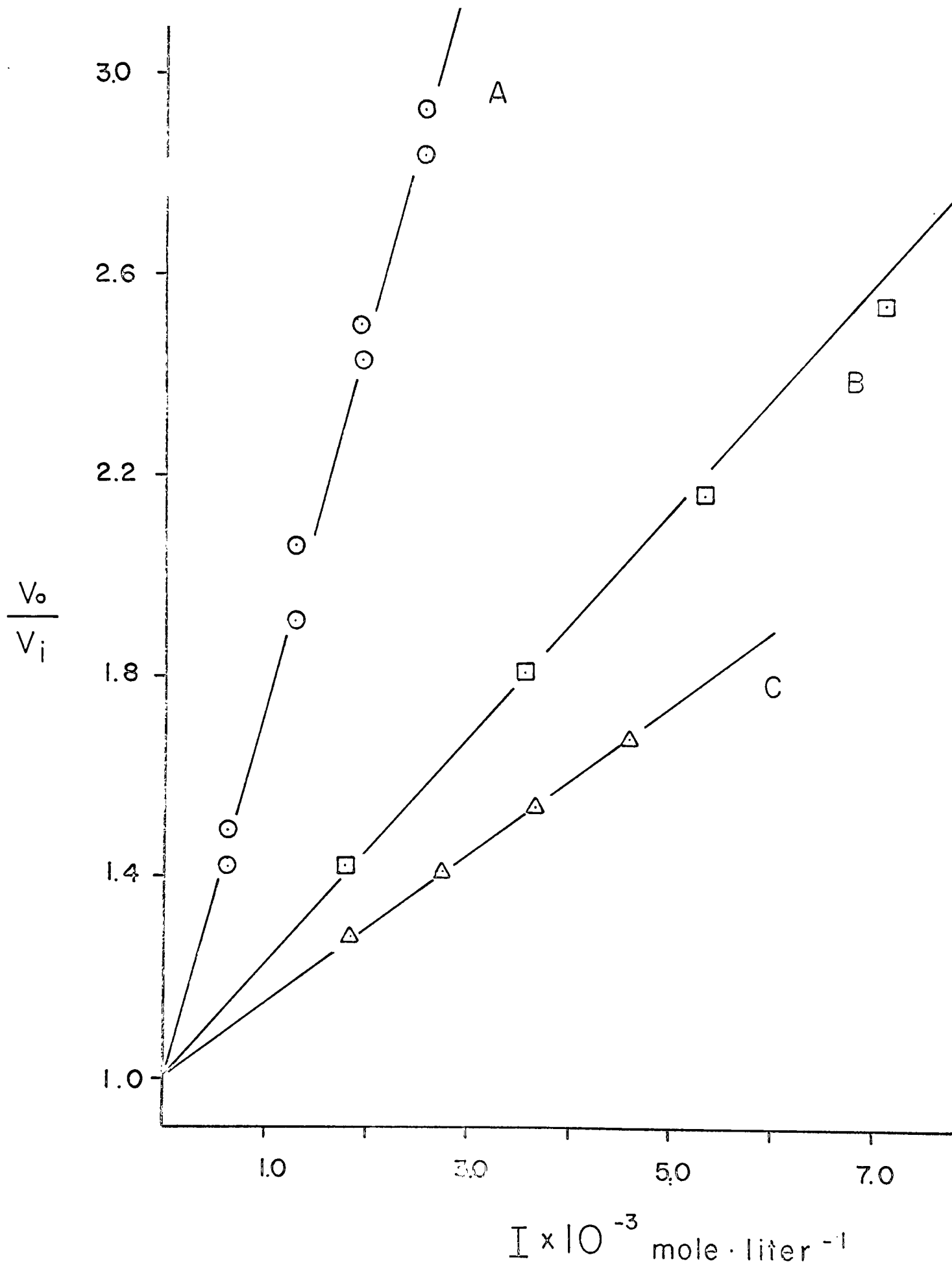
Figure 3

Variation with temperature of the inhibition of ACh
hydrolysis by AChE, for two structurally different
competitive inhibitors of the enzyme

CURVE A: phenyl trimethyl ammonium iodide (compound N° 12)
at 30°C

CURVE B: D-(-)- β -methyl acetyl choline iodide (compound N° 21)
at 10°C

CURVE C: D-(-)- β -methyl acetylcholine iodide (compound N° 21)
at 25°C



b) L-(+)- β -methyl acetylcholine (compound N^o 20):

The Michaelis constant for this substrate was graphically determined at five different temperatures by the method of Eadie (Figure 4, page 52). The variations of K_m and V_m with the temperature are given in Table III. This ester has been checked for spontaneous hydrolysis in aqueous solutions and was found to be highly stable, since no hydrolysis could be detected over periods of ten days in water.

c) Formylcholine:

Attempts to evaluate the K_m of formylcholine iodide under conditions similar to those applied to ACh, were unsuccessful, owing to the rapid and spontaneous rate of hydrolysis of this compound in aqueous solutions. Under those conditions, the spontaneous hydrolysis was so large when compared to enzymic hydrolysis, that the evaluation of the enzyme's contribution to total hydrolysis was subject to very large errors. Nevertheless, the rate constant for the pseudo first order aqueous hydrolysis of this ester has been determined at three temperatures so as to obtain the energy of activation for this reaction.

Figure 4

Eadie's plots at three different temperatures for the
substrate L-(+)- β -methyl acetyl choline

Each point represents an experimental measurement.

The slope is equal to $-K_m$. Complete data
are given in Table III.

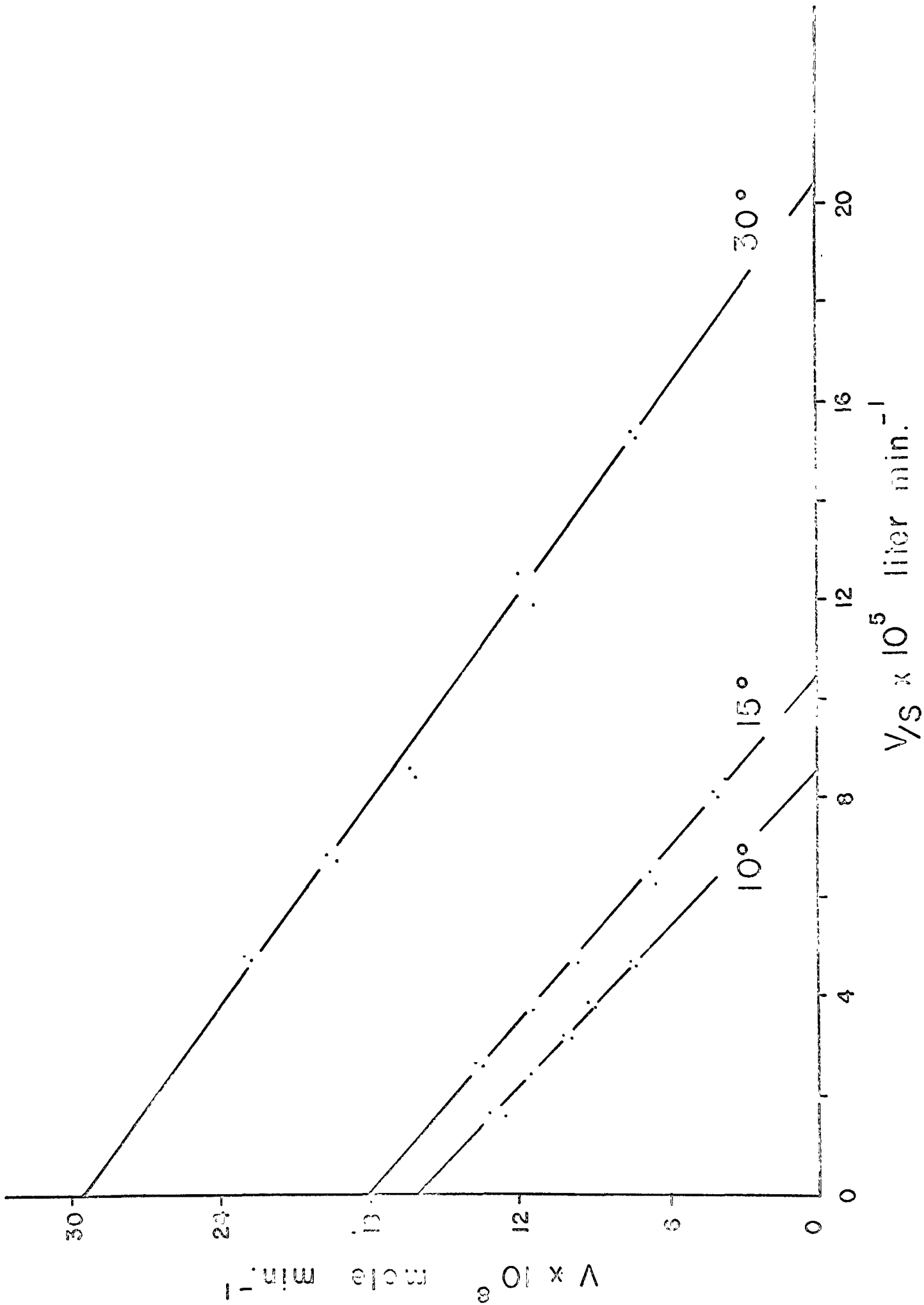


TABLE III

Variation of K_m with temperature for the
hydrolysis of L(+)- β -methylacetylcholine a)

$T^{\circ}C$	V_m a) $\times 10^8$ mole.min ⁻¹	V_m/K_m $\times 10^5$ liter.min ⁻¹	K_m b) $\times 10^3$ mole.liter ⁻¹
10°	16.1	8.0	1.87
15°	18.1	10.3	1.77
20°	21.8	13.2	1.65
25°	25.9	16.7	1.55
30°	29.5	20.4	1.44

a) when the enzyme concentration is 10 units in 25 ml of reaction medium

b) here, K_m is the apparent Michaelis constant

PSEUDO FIRST ORDER RATE CONSTANT FOR THE
SPONTANEOUS HYDROLYSIS OF FORMYL CHOLINE IN WATER

<u>T°C</u>	<u>k (pseudo first order)</u>
20°C	$1.1 \times 10^{-3} \text{ min}^{-1}$
25°C	$2.2 \times 10^{-3} \text{ min}^{-1}$
30°C	$4.4 \times 10^{-3} \text{ min}^{-1}$

The energy of activation is ~ 26 Kcal/mole

3) Arrhenius plots:

The effects of temperature on K_i give rise to linear Arrhenius plots. Typical results are illustrated in Figure 5, (page 55), where each compound is illustrative of a class.

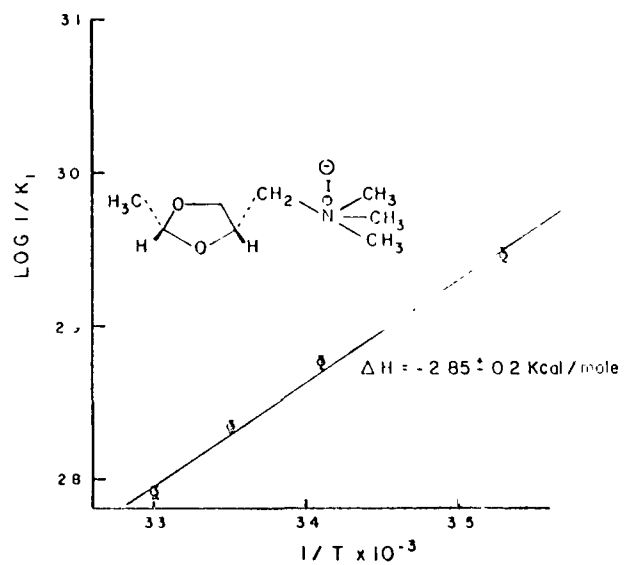
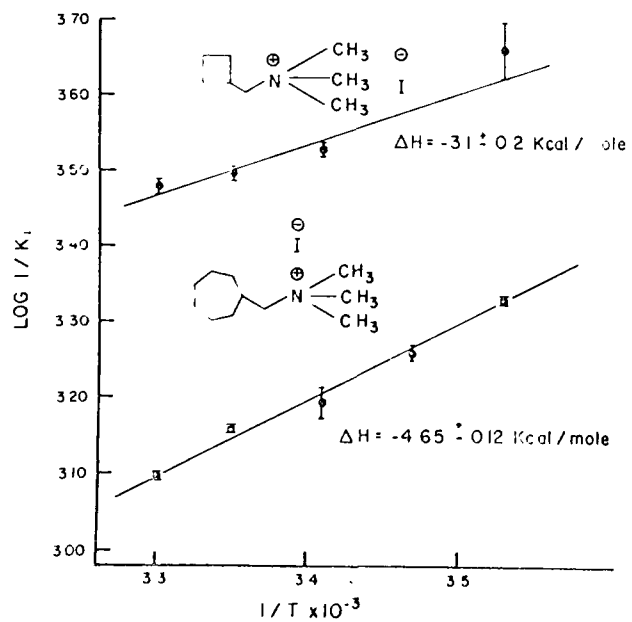
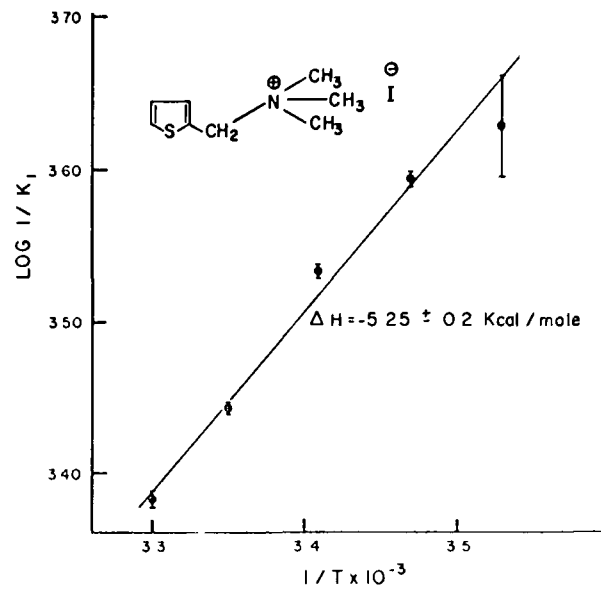
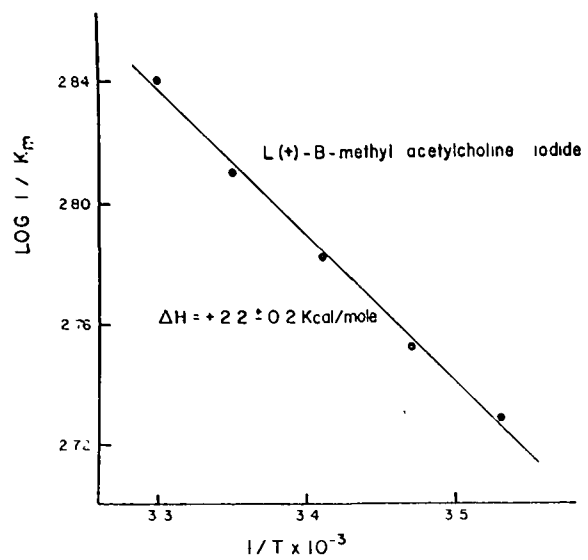
4) Thermodynamic Quantities:

These parameters are assembled in Table IV and the deviations resulting from experimental errors are also given. One can readily note the constancy of ΔF for the entire series of 23 ligands (series D of Table V). The mean value for the free energy of binding is equal to

Figure 5

Arrhenius plots for four inhibitors of AChE

each being representative of a class of compounds, and
for the substrate L-(+)- β -methyl acetyl choline (where the
apparent Michaelis constant is treated
as an equilibrium constant).



-4.2 ± 0.6 Kcal./mole. However, some special trends are operative and serve to characterize compounds with similar structures. For example, the oxygen-containing compounds like the 1,3-dioxolane derivatives and the ester compounds (series C of Table V) have a somewhat lower mean ΔF value of -3.8 Kcal./mole. In contrast, the cyclo alkyl derivatives of TMA and the aromatic compounds (series B of Table V) have a more negative $\overline{\Delta F}$, the average value being -4.5 Kcal./mole.

The enthalpy and entropy terms however, are subject to very large inflections, even among compounds of similar structures, but owing to compensation effects, these variations do not affect the free energy quantities. To illustrate this observation, it will suffice to consider the case of the four ligands carrying an aromatic substituent (compounds N° 9 to N° 12) where the $\delta \Delta H$ of the substituent can differ by some 5 Kcal./mole and the $\delta \Delta S$ by ~ 17 e.u. when comparison is made between the thienyl and furyl substituents. Note however, that the $\delta \Delta F$ for the same pair of compounds is only 0.1 Kcal./mole. Variations of a similar magnitude in ΔH and ΔS are observed between pairs of oxygen-containing ligands and within the cyclo alkyl TMA series of derivatives. Within a series of chemically related compounds, the invariability of ΔF is nevertheless rigorously respected.

The thermodynamic parameters indicate that the binding of the thienyl differs markedly from those of similar chemical structures such as furyl and phenyl rings. Important differences in the interaction me-

TABLE IV

Thermodynamic quantities for the binding of inhibitors
and substrates to AChE

Compound No	TMA Derivative	ΔF 25° Kcal./mole	ΔH Kcal./mole	ΔS e.u.
1	cyclobutyl	-4.75 ± 0.12	-3.1 ± 0.2	+5.6 ± 0.5
2	cyclopentyl b)	-4.79 ± 0.16	-3.0 ± 0.7	+5.9 ± 1.4
3	cyclohexyl	-4.34 ± 0.12	-0.85 ± 0.5	+11.7 ± 1.0
4	cycloheptyl	-4.29 ± 0.09	-4.65 ± 0.12	-1.3 ± 0.25
5	cyclo octyl	-4.44 ± 0.16	-5.0 ± 0.5	-1.9 ± 1.1
6	bicycloheptyl	-4.11 ± 0.09	0.0 ± 0.3	+13.8 ± 0.7
7	bicyclo octyl	-4.11 ± 0.11	+0.2 ± 0.2	+14.6 ± 0.5
8	tricyclodecyl (adamantyl)	-4.25 ± 0.08	-3.0 ± 0.3	+4.2 ± 0.6
9	2-furyl	-4.80 ± 0.14	-0.35 ± 0.3	+14.9 ± 0.7
10	5-methyl-2-furyl	-4.46 ± 0.16	-1.4 ± 0.3	+10.4 ± 0.7
11	2-thiophenyl	-4.69 ± 0.11	-5.25 ± 0.2	-1.9 ± 0.5
12	phenyl	-4.60 ± 0.13	-0.51 ± 0.2	+13.6 ± 0.5
13	4-L(+)-1,3-dioxolane	-3.98 ± 0.09	-6.4 ± 0.2	-8.1 ± 0.5

Table IV continued

14	4-D(-)-1,3-dioxolane	-3.78 ± 0.07	-2.95 ± 0.15	+2.7 ± 0.4
15	4-L-cis-2-methyl-1,3-dioxolane	-4.52 ± 0.17	-1.3 ± 0.3	+10.8 ± 0.8
16	4-L-trans-2-methyl-1,3-dioxolane	-3.73 ± 0.10	-2.75 ± 0.55	+3.3 ± 1.0
17	4-D-cis-2-methyl-1,3-dioxolane	-3.85 ± 0.07	-2.85 ± 0.20	+3.36 ± 0.45
18	4-D-trans-2-methyl-1,3-dioxolane	-3.76 ± 0.08	-2.84 ± 0.20	+3.1 ± 0.5
19	2-1,3-dioxolane (isodioxolane)	-4.24 ± 0.13	-1.4 ± 0.4	+9.6 ± 0.9
20	L(+)-β-methyl acetylcholine ^{ac}	-3.82 ± 0.12	+2.2 ± 0.2	+20.2 ± 0.5
21	D(-)-β-methyl acetylcholine ^a	-3.58 ± 0.01	-4.56 ± 0.05	-3.29 ± 0.25
22	β-trimethylammonium methyl propionate ^{ab} (reversed ACh)	-3.28 ± 0.11	-4.6 ± 0.4	-4.3 ± 0.8
23	acetylcholine (ACh) ^{ac}	-4.74 ± 0.02	0	+15.9 ± 0.1
25(C ₆)	n-hexyl	-4.06 ± 0.08	-3.9 ± 0.2	+0.54 ± 0.5
26(C ₁₁)	n-undecyl	-5.56 ± 0.18	-2.4 ± 0.2	-10.6 ± 0.6

a) Full name is given

b) At 30°C

c) The K_m constant for those two substrates has been assumed to be equal to the equilibrium constant of binding; the ΔF and ΔH quantities have been evaluated consequently. The questionability of doing so will be discussed later. The thermodynamic quantities have been defined as apparent ΔF , ΔH and ΔS . (see page 43,44).

TABLE V^{*}

Relation between $\overline{\Delta F}$ and intercepts on ΔH axis
as applying to each group of ligands

	Intercept of L_H on ΔH axis Kcal./mole	Intercept of L_S on ΔH axis Kcal./mole	Average free energy quantity $\overline{\Delta F}$ Kcal./mole
GROUP A (22 pairs)	-4.15	-4.09	-4.19
GROUP B (12 pairs)	-4.54	-4.50	-4.47
GROUP C (10 pairs)	-3.82	-3.80	-3.85
GROUP D (23 pairs)	-4.15	-4.09	-4.21
GROUP E (11 pairs)	-3.84	-3.81	-3.93

* all values are given in Kcal./mole

L_H is the regression line of ΔS on ΔH

L_S is the regression line of ΔH on ΔS

chanism of the enzyme with diastereoisomers of the 1,3-dioxolane series and the enantiomeric D-(-)- and L-(+)- β -methyl acetyl choline are also operative.

Note worthy is the case of the n-undecyl derivative (compound N^o 26), where it is observed that ΔF assumes a much larger value (when compared with the n-hexyl analog for example) than expected, thus indicating a departure from the general mechanism of interaction which is characteristic of the short chain n-alkyl derivatives of TMA. The value of ΔF here, is more negative than the mean value of 4.2 Kcal./mole by 1.4 Kcal.

Among the eight cyclo alkyl ligands derivatives of TMA, there is a tendency for ΔH and ΔS to become more positive when the molar volumes are increased (Tables I and IV). However, very significant departures from this additivity rule are observed in the case of the cycloheptyl, cyclooctyl and adamantyl ligands, which exhibit much lower enthalpy and entropy values by comparison with expectations based on the behavior of the smaller ring analogs.

A glance at Tables II and IV reveals that no relationship can possibly exist between the magnitude of the inhibition constants and the thermodynamic parameters for the binding: ligands with dissimilar structures like the 2-thienyl-TMA and D-(-)- β -methyl acetyl choline have respectively one of the largest and one of the smallest inhibition constants of the whole series, although their thermodynamic parameters are

comparable. The same applies to the bicycloheptyl and 2-furyl derivatives. However, especially in the cyclo alkyl series, one may find some parallelism between the thermodynamic parameters and the inhibition constants. This seems to apply to homologous compounds like the pairs cyclo butyl-cyclopentyl, cyclo heptyl-cyclooctyl and bicyclo heptyl-bicyclooctyl.

5) Statistical Treatment of the Data:

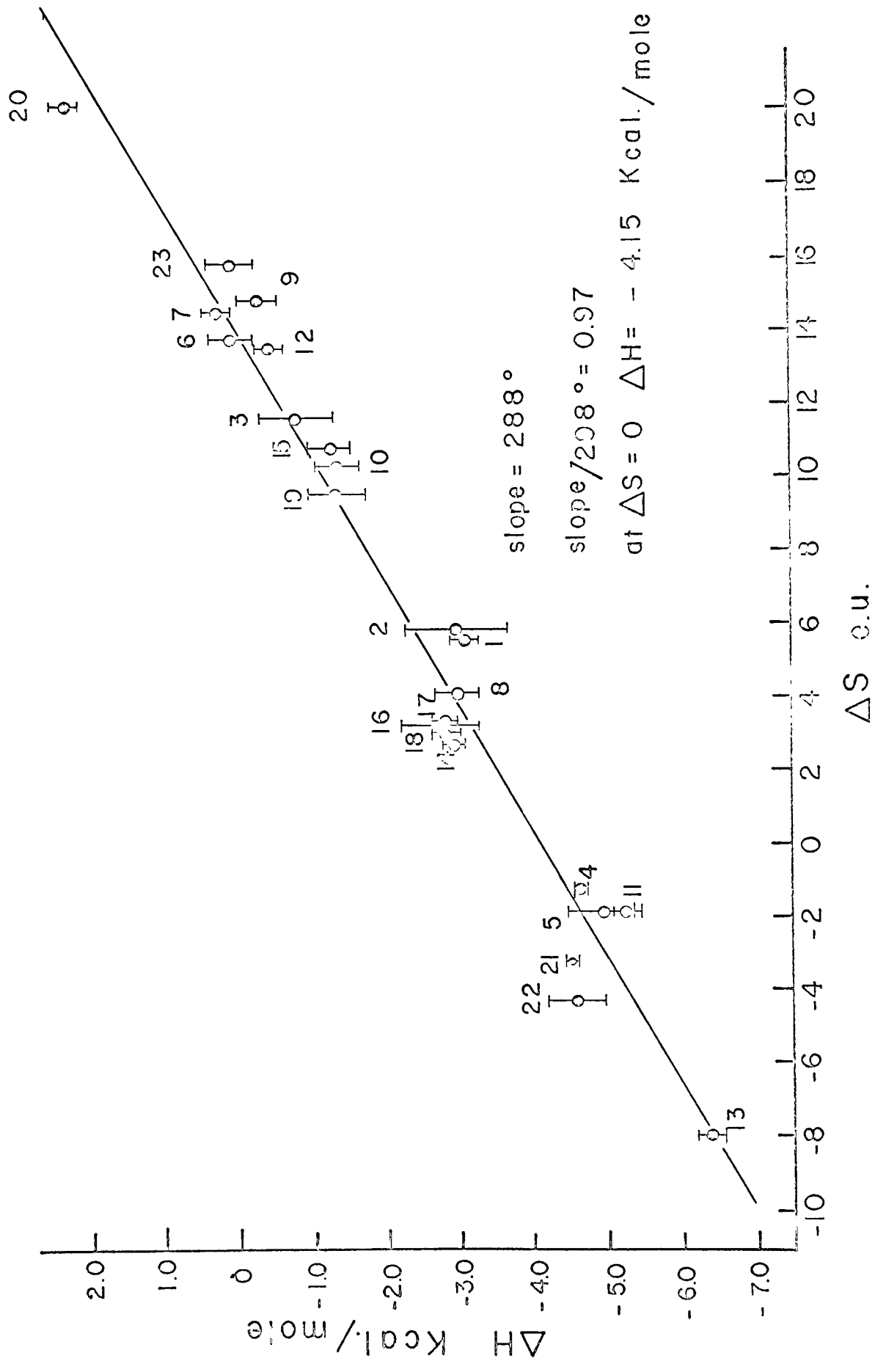
According to our presentation on the enthalpy-entropy relationships (see Chapter I), it is essential to verify the actual existence of additivity relationships between ΔH and ΔS when a series of chemical events of the same physical nature are considered. Here, we have access to the thermodynamic parameters that are characteristic of the binding equilibria with AChE for a series of twenty-three ligands, all of which are known to interact with the same site since they all possess a common cationic moiety. The various possible "pairs of observation" on ΔH and ΔS (data of Table IV) are assembled in Figure 6 (page 62). One notes first that the "pairs" have ΔH and ΔS values that are spread over a considerable range, with a maximum $\delta \Delta H$ of $\sim 8.6 \text{ Kcal./mole}$ and a $\delta \Delta S$ of some 30 e.u. Should one compare these variations with those reported (93, 94) for interactions of a different nature, the conclusion is obvious that the observed ranges in the variation of ΔH and ΔS are quite large indeed.

Figure 6

Isoequilibrium plot for the thermodynamic parameters
relative to the binding of the inhibitors and substrates
listed in Table IV

(excepting compounds N° 25 and N° 26). The slope and intercept were evaluated by the application of the least square method of fitting. The regression line of ΔH on ΔS (see Table V) is shown.

Note: For reasons of clearness in the presentation, the uncertainty in the evaluation of the thermodynamic parameters is given only for the ΔH coordinate. But there exists also an error of same order of magnitude in the evaluation of ΔS . Therefore, as was pointed out by Grunwald and Loeffler (94), the probable error contour for a given pair of ΔH and ΔS values has the shape of an elongated ellipse, since the absolute error in ΔS is a multiple of that for ΔH .



As seen in Chapter I, the operation of a single physical mechanism to a series of reactions is a valid conclusion when a linear isoequilibrium relationship actually exists. Thus, prior to the proposal of a mechanism of interaction for a complete series of ligands with the enzyme AChE, it is an important prerequisite to establish the linearity of the relationship between the ΔH and ΔS parameters for the interactions.

The correlation coefficient is indicative of the degree of validity of a linear relationship. Therefore, the calculation of this coefficient must be done first, after which a least square fit of the data can be applied to characterize the curve.

The correlation coefficient r , was calculated according to the following expression

$$r = \frac{(\sum H'S')^2}{\sum(H')^2 \sum(S')^2} \quad (97) \quad [1]$$

where

$$\sum H'S' = \sum(\Delta H\Delta S) - \frac{(\sum \Delta H)(\sum \Delta S)}{n}$$

$$\Sigma (H')^2 = \Sigma (\Delta H)^2 - \frac{(\Sigma \Delta H)^2}{n}$$

$$\Sigma (S')^2 = \Sigma (\Delta S)^2 - \frac{(\Sigma \Delta S)^2}{n}$$

with

$$\Sigma \Delta H = -54.36$$

$$\Sigma (\Delta H)^2 = 228.76$$

$$\Sigma \Delta S = +143.0$$

$$\Sigma (\Delta S)^2 = 2152.67$$

$$\Sigma (\Delta H \Delta S) = +12.24$$

and taking n as being 23, (Table IV with N^o 25 and N^o 26 excluded).

The sums are always for the 23 values and the ΔH and ΔS terms are taken from Table IV.

From [1], r was found to be equal to 0.98 (for series D of Table V).

Such a value for r, justifies the application of a linear relationship between ΔH and ΔS . A least square fit may then be applied to the pairs (ΔH , ΔS) of data in Table IV, in order to characterize the curve. Previously, groups of ligands from Table IV were assembled according to their structures, and two curves were constructed for each group; one curve called

L_S being the line of regression of ΔH on ΔS and the other, L_H , being the line of regression of ΔS on ΔH . Each line is characterized by a slope m and one intercept. The ligands were classified as follows:

- GROUP A - (22 pairs) - includes all the ligands of Table IV, with the exception of ACh (N^o 22), n-hexyl (N^o 25) and n-undecyl (N^o 26)
- GROUP B - (12 pairs) - includes ligands that are either cyclo alkyl derivatives of TMA, or that include an aromatic substituent on TMA.
- GROUP C - (10 pairs) - for the 1,3-dioxolane derivatives, the reversed ester of ACh and the β -methyl acetylcholines.
- GROUP D - (23 pairs) - is the same as GROUP A + ACh
- GROUP E - (11 pairs) - is the same as GROUP C + ACh

The slopes and intercepts of the lines corresponding to each group are presented in Table VI.

The statistical treatment of the data indicates that the square root of the product of the slopes of two regression lines gives the correlation coefficient (99); the coefficient for each group is given in Table VI.

It is readily seen that the slopes and intercepts of the two regression lines in any single group taken from Table VI are very similar.

TABLE VI

Least square curve fitting of two regression lines and correlation coefficients

	L_H (regression line of ΔS on ΔH)			L_S (regression line of ΔH on ΔS)			$r = \sqrt{m_H m_S}$
	m_H	Intercept on ΔS axis	Intercept on ΔH axis	m_S	Intercept on ΔS axis	Intercept on ΔH axis	(Correlation coefficient)
GROUP A (22 pairs)	0.00345	-14.3	-4.15	280°	+14.6	-4.09	0.98
GROUP B (12 pairs)	0.00325	+14.8	-4.54	302°	+14.9	-4.50	0.99
GROUP C (10 pairs)	0.00351	+13.4	-3.82	280°	+13.6	-3.80	0.99
GROUP D (23 pairs)	0.00349	+14.5	-4.15	288°	+14.8	-4.09	0.98
GROUP E (11 pairs)	0.00363	+13.9	-3.84	270°	+14.1	-3.81	0.99

m_H = slope of L_H (in reciprocal degrees)

m_S = slope of L_S (in absolute degrees) > with $m_H = 1/m_S$ (theoretically)

intercept on the ΔS axis is given in e.u.

intercept on the ΔH axis is given in Kcal./mole

r is the correlation coefficient

Such behavior is a direct consequence of the existence of correlation coefficients close to unity in all cases.

Tables of correlation indicate that for a number $N = 20$ pairs of observation, the probability of obtaining by chance a value of r equal to 0.68 is 0.001 (100). The results presented above are then indicative of a true linear relationship between ΔH and ΔS and this remains valid regardless as to whether the series of TMA derivatives is considered as a whole or as separate classes of ligands.

It should be noted that although the correlation is just as good for compounds of group B, as it is for those of group E, the curves exhibit slightly different characteristics, but which represent only a minor departure from the main curve (that of group D). Although this point will be analyzed in greater detail in the next chapter, note should be taken here, that a true relationship exists for each group of compounds (as classified above) between the intercepts of the curves on the ΔH axis and the mean value of the corresponding free energy quantities $\overline{\Delta F}$ for the same group of compounds. These relationships are clearly shown in Table V. This point is of importance and allows some basic conclusions that will be developed later.

6) The Isoequilibrium Plot:

As the above analysis indicates, one can establish a linear relationship (Figure 6, page 62) between ΔH and ΔS for the entire series

(group D of Table VI) of inhibitors studied (n-hexyl and n-undecyl are not included). This line L_H for which a correlation coefficient of 0.98 was established, has a slope of 288° and an intercept on the ΔH axis of -4.15 Kcal./mole . In spite of the high significance of the correlation coefficient, small departures from linearity exist, but in no way, can the validity of the isoequilibrium relationship be questioned, using small deviations as a basis, since they can be ascribed to minor perturbing mechanisms that are superimposed on the major linear relationship. Thus, a linear relationship of the form $\Delta H = \beta \Delta S$ would apply for this series of ligands where β , the slope in absolute degrees, may be considered as equal to the experimental temperature, 298° . The existence of this relationship is clearly indicative of the operation of a single physical mechanism of interaction for the entire series of ligands.

In addition, the Figure 6 (page 62) shows that the interactions of the substrates ACh (compound N^o 23) and L(+)- β -methyl acetylcholine (compound N^o 20) can also be described by the same general physical mechanism, although in those cases steady-state constants ($K'_{m,s}$ are apparent Michaelis constants) instead of true equilibrium constants ($K_{i,s}$) have been used. As will be shown later, the applicability of K_m values in our thermodynamic studies gives an indication that they can be treated as real equilibrium constants for all practical purposes.

Figure 7 (page 70) has been included to show that the relationship that was established for the various ligands assembled in Table IV, hold just as well for the series of n-alkyl-TMA derivatives, whose thermodynamic parameters for the same reaction have been determined previously by Belleau, Tani and Lie (83) (Table VII). The plot of Figure 7 is the same as that shown in Figure 6 and it can be seen that the points follow the same general relationship very closely. The proposal was made (95) that these compounds interact with the AChE surface by the same physical mechanism. Indeed, Figures 6 and 7 indicate that this general mechanism also applies to ligands that are not n-alkyl-TMA derivatives. Figure 7 also includes the ΔH and ΔS quantities for the longer chain ligand n-undecyl-TMA (C_{11}), in which case a significant departure from the linear relationship is observed, and also for the n-hexyl-TMA (C_6) ligand.

7) Effects of Structure on the Enthalpy of Binding:

Following the observation that a large number of ligands interact with the AChE surface by the same physical mechanism, the structural features of these ligands, as they influence the thermodynamic parameters that characterize the mechanism, will now be analyzed. It can be emphasized again that a change in the structure of the ligands does not change the basic physical mechanism of interaction, as is clearly demonstrated by the existence of an isoequilibrium relationship (see Figure 6). The

Figure 7

Isocquilibrium plot similar to that shown on Figure 6

Here is shown the applicability of the same linear relationship to the binding of a few n-alkyl derivatives of TMA (see Table VI). Also included, are the thermodynamic parameters for compounds N^o 25 (C₆) and N^o 26 (C₁₁) (see Table III)

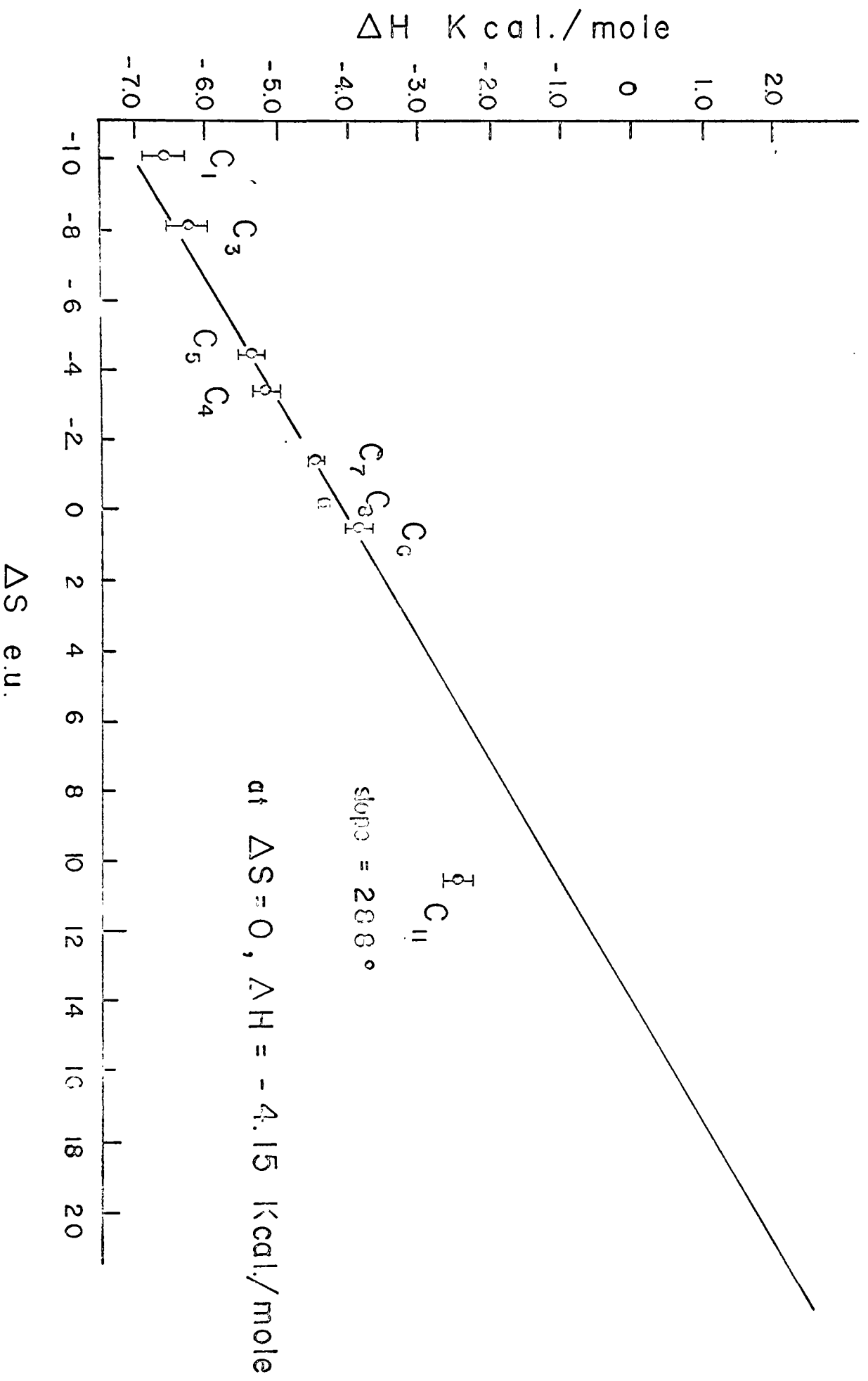
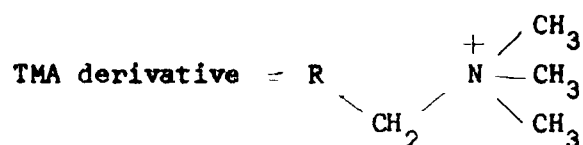


TABLE VII
Thermodynamic quantities* for the binding of
n-alkyl TMA derivatives on AChE



Compound No	R	ΔF 25° Kcal./mole	ΔH Kcal./mole	ΔS e.u.
C ₁	H	-3.59 ± 0.02	-6.60 ± 0.30	-10.1 ± 1.0
C ₃	C ₂ H ₅	-3.92 ± 0.01	-6.32 ± 0.30	-8.1 ± 1.0
C ₄	C ₃ H ₇	-4.20 ± 0.01	-5.22 ± 0.22	-3.4 ± 0.7
C ₅	C ₄ H ₉	-3.76 ± 0.01	-5.40 ± 0.20	-5.5 ± 0.7
C ₇	C ₆ H ₁₃	-4.08 ± 0.01	-4.49 ± 0.01	-1.4 ± 0.4
C ₈	C ₇ H ₁₅	-4.34 ± 0.01	-4.40 ± 0.05	-0.2 ± 0.17

* (data taken from Belleau and Tani (83))

structural changes in the ligands merely serve to cause the points to move only on the curve. But such changes as the removal of electron-rich centers or the addition of functional groups of high reactivity may have been expected to perturb the fundamental mechanism, so that deviations from the isoequilibrium plot would have been observed.

However, for several series of ligands, the common TMA moiety is substituted by groups that are physically very similar, which limits the importance of secondary perturbing mechanisms. In this respect, when going from formyl to acetylcholine or from acetyl to propionyl choline, only the addition of a methylene group is involved in the structural change, a modification of minor importance. Any thermodynamic inflection in their respective interaction mechanism will be indicative of a specificity effect if the deviation is out of line with normal expectations.

Modifications of structure, will sometimes produce specific effects on binding with the enzyme, and such may be anticipated and explained without recourse to thermodynamic data. For example, interchanging the position of the carbonyl group in acetyl choline with the ether oxygen, to give reversed ACh (compound N^o 22), results in the formation of a weak inhibitor devoid of substrate properties. At the same time, ΔH binding decreases by some 4.5 Kcal./mole. The homologous substrate L(+)- β -methyl acetyl choline has a K_m value five times as large as that for ACh; in this case the apparent ΔH binding amounts to some

2 Kcal./mole. Changing the configuration so as to obtain the D-(-)- β -methyl acetylcholine results in a change from a substrate to an inhibitor; this change is reflected in a $\delta \Delta H$ of some 7 Kcal./mole. ($\delta \Delta H$ binding is of course linearly related to $\delta \Delta S$). In these examples, the effects of structural changes are directly reflected in a kinetic property of the system.

On the other hand, the importance on binding of structural modifications such as those which characterize the 1,3-dioxolane series of isomeric dioxolanes (compounds 13 to 19) can be appreciated only by analyzing the effects of configuration on the thermodynamic parameters. The stereoisomers 4-trimethyl ammonium methyl-D(-) and L(+)-1,3-dioxolane are characterized by a change in their respective ΔH binding of some 3.5 Kcal./mole. Addition of a *cis*-methyl group in the 2-position of the L(+)-dioxolane (compound 13) results in an increase in ΔH binding of ~ 5 Kcal./mole. No such effect exists in the D-series of enantiomers, since the binding of the three isomers of this series (compounds NOS 14, 17, 18 on Figure 6), is characterized by equivalent thermodynamic constants. Complex formation with the optically inactive isodioxolane (compound 19), which formally results from a transposition of the TMA substituent, leads to thermodynamic parameters that markedly differ from those of the D(-) and L(+)-isomers, the difference in ΔH with the former being 1.5 Kcal./mole and with the latter 5 Kcal./mole.

The isosteric change furan to thiophene, has no influence on the free energy quantities, yet it results in an important modification (with a $\delta \Delta H$ binding of 5 Kcal./_{mole}) in the mechanism of interaction with the AChE surface. Here, the enzyme exhibits an unexpected sensitivity towards these hetero aromatic rings.

The flexible ester ligands are situated at extreme positions on the isoequilibrium curve; the non-substrate ones (compounds N^o 21, N^o 22) have strongly negative ΔH values, while the substrate-esters, ACh and L(+)- β -methyl ACh give rise to large positive increments in ΔH .

When the n-alkyl chains are constrained by cyclization to 4-, 5- or 6-membered rings (compounds N^o C₄ and C₅ of Table VII and N^o 26 of Table IV), a constant effect on ΔH is observed. The increase in ΔH binding upon cyclization of these three alkyl chains averages 2.5 Kcal./_{mole}. However, the behavior of larger cycles like cycloheptyl and cyclooctyl is in sharp contrast, since these ligands exhibit ΔH values that are characteristic of the open-chain analogs C₇ and C₈. The flexibility of these large rings allows for a great deal more internal freedom than the smaller rings, which explains their ΔH characteristics. When rigidity is maintained in these cyclo alkyl rings, as in the case of the bicycloheptyl and bicyclooctyl derivatives, ΔH binding is comparable to that for the cyclohexyl ring. On the basis of these considerations on the rigidity and flexibility of cyclic alkanes, one would expect the highly-rigid structure of adamantane to behave like the bicyclic-analogs.

However, its position on the isoequilibrium plot is anomalous (compound N° 8), which suggests that a special effect is operative in this case. The expected position of adamantane on the curve of Figure 6, is some 3 Kcal./_{mole} higher than the experimental ΔH value.

CHAPTER IV

DISCUSSION

A) The Size of ΔF :

The appearance of the isoequilibrium plot of figure 6 (page 62) suggests that a common physical mechanism of interaction with the AChE surface applies to all ligands examined in this study. This statement is supported by the fact that the thermodynamic relationships satisfy all the afore mentioned requirements for a unique mechanism, namely the correlation coefficient which confirms the linearity of the relationship and the range of values covered by the ΔH and ΔS parameters which eliminates the possibility of a fortuitous identity of β with T_{exp} . On the basis of the observation that the TMA moiety contributes a constant effect, it follows that the physical mechanism of interaction of the ligands concerns only the substituents of the TMA heads.

The compensation effects observed in the relative values of ΔH and ΔS for each pair of observations, accounts for the constancy of ΔF at 4.2 ± 0.6 Kcal./mole (see data of group D of Table V). It is interesting to compare this average value of ΔF with that for other processes where similar compensation effects are also operative. Jeffrey and Coates reported the following values for the self-association of bovine insulin (134).

	ΔF	ΔH	ΔS
	Kcal./mole	Kcal./mole	e.u.
dimerization	-5.4	- 7.1	-5.5
tetramerization	-3.9	-16	-41
hexamerization	-3.8	49	177

The fluctuations in ΔF are quite small when compared with the very large variations in both the enthalpy and entropy terms. (It is noticeable that two processes involving different molecular species, such as the reversible association of protein subunits and the binding of small ligands to an enzyme molecule, should be characterized by a comparatively small free energy of association averaging about 4 Kcal./mole. This suggests that the compensation effects between ΔH and ΔS may have a common origin).

However, it is evident that compensated forces cannot contribute to the binding energy of the ligands with the enzyme. On the basis of the iso equilibrium relationship, $\Delta H = \beta \Delta S$, the non-compensated contribution of ΔH to ΔF can be obtained by setting ΔS equal to zero; the fundamental and constant force contributing to the observed ΔF value can thus be evaluated. The ΔH -intercept as evaluated by the least-square

method was found to be -4.15 Kcal./mole (see Table V). This value is equal for all practical purposes to ΔF throughout the series.

Some time ago, Hepler (135) has introduced the concept of internal and external contributions to the thermodynamic parameters characterizing the process of ionization of organic acids in water; the respective contribution arising from the interaction of the acid and the anion and that originating from environmental effects such as the solvent were separated and treated as additive functions. Accordingly, the thermodynamic quantities reported in Table IV must also be considered as being made of two parts: a) the quantities $\Delta H_{\text{internal}}$ and $\Delta S_{\text{internal}}$ which account for the interaction enzyme-ligand and b) the $\Delta H_{\text{external}}$ and $\Delta S_{\text{external}}$ components originating from the interactions of the enzyme and the ligands with water.

In the case of the AChE-TMA ligands interactions, the $\Delta H_{\text{internal}}$ term (4.15 Kcal./mole) accounts completely for the constant ΔF average value; therefore, the compensation effects forming the basis of the iso-equilibrium plot must be concerned exclusively with the external components, that is with the interactions of the cosolutes with the solvent. To confirm this, statistical computations of the slopes and intercepts of various plots were applied using smaller assemblies of data, the choice of which being dictated by the constancy of certain structural features between families of ligands. A glance at Table V (page 59) shows that if, for example, the 1,3-dioxolane derivatives and the ester ligands are taken as a separate group (group C), the estimated value of $\Delta H_{\text{internal}}$ is 3.80 Kcal./mole; the average ΔF ^{for} this group is 3.85 Kcal./mole. A similarly

close agreement between $\Delta H_{\text{internal}}$ and average ΔF values is also observed for the group of the cycloalkyl-TMA ligands; in this case, $\Delta H_{\text{internal}} = \Delta F = 4.5$ Kcal./mole. This also applies to group A, which comprises the thermodynamic data for all the ligands minus ACh. Such a close agreement cannot possibly be due to a fortuitously favorable selection of ligand groups, which confirms that the effect of the interactions with the environment is internally cancelled and thus cannot be reflected in the value of ΔF .

The first significant conclusion from the molecular point of view that the above facts permit consists in that the value of $\Delta H_{\text{internal}}$ (that is the uncompensated component of ΔF) must reflect the interaction between the TMA moieties with the anionic site of the AChE active center, where the cation is held by coulombic forces. It is very nearly the same for all the ligands, thus showing beyond any question that the ΔF parameter is insensitive to structural changes, by comparison with the enthalpy and the entropy parameters; hence the ΔF parameter can only account for the ion-ion interaction force.

The second conclusion that can be drawn, concerns the specificity parameter which is reflected only in structural alterations of the "environment". In fact, the slope β for the isoequilibrium relationship has the highly characteristic value of 288° , which suggests that the thermodynamically significant reaction concerns the perturbation of the structure of the water associated with the reactants (94). A compa-

risson of the thermodynamic data for the tetramethylammonium ion on the one hand, and of its derivatives on the other, shows that the introduction of any substituent on this ion always contributes to making both the ΔH and ΔS values more positive; this is true for all the cases so far investigated. Hence, this increased positiveness as caused by any substituent must be associated with the melting of water molecules on the enzyme surface especially around the active sites.

According to Kauzmann (30), the slope β for any transfer process where the breaking of "iceberg structures" surrounding hydrocarbon molecules in water is believed to take place, (such as is the case in the transfer of non-polar solutes from water to non-polar solvents) ranges between 50° and 150° . The value of β in our case is 288° , which is very close to that expected for a process where ice-melting controls the thermodynamic parameters (30). A similar value has been reported for the formation of gas hydrates derived from methane, ethane, and propane (136). This is an indication that in the case of ligand binding on AChE, the extruded water must be a structurally important component of the protein molecule. This conclusion supports the recent proposal by Grant (137), who deduced on the basis of dielectric measurements, that the structure of protein-bound water is more "... ice-like rather than akin to liquid water".

It can be seen then that in the case of the binding of small ligands to the active center of AChE, specificity effects must be analyzed in terms of the removal of bound water molecules, forming an integral part of the protein molecule.

B) The Binding Equilibrium and the Removal of Water Molecules:

The theory of extrathermodynamic relationships as applied to the system AChE-TMA ligands, supports an interaction mechanism, which shall be referred to as the water extrusion hypothesis. (It would be useless here to ascertain the obvious occurrence of bound water molecules on the surface of proteins). Consequently the removal, from the protein surface, of "structurally bound water", will naturally be reflected in the value of the slope β . However, such a statement will be valid only if the removal of the water molecules is the actual parameter affecting in a determinant manner the energetics of the binding process. In other words, the activation energy for the binding process must be controlled by the extrusion of water molecules as the limiting step if the thermodynamic parameters are to reflect changes primarily in the structure of these molecules.

Direct evidence that the removal of water molecules from binding surfaces can be rate-limiting for addition-complex formation cannot be ascertained by the methods applied here. However, that this expectation is probably valid is supported by a number of known facts that are very intimately related to the problem under consideration. Pertinent to our problem is the recent work of Molyneux and Frank on ligand binding onto polyvinylpyrrolidone(139) and that of Cramer et al (140) on the formation of inclusion compounds with α -cyclodextrins. According

to these works, the process of complex formation between ligands and a macromolecular component such as a protein, may be dissected into a series of discrete consecutive steps, as follows: step 1) the melting of water molecules surrounding the ligand; step 2) the melting of water in the region of the binding sites on the macromolecule; step 3) the chemical interaction between the ligand and the macromolecule; step 4) the reformation of an order ^{ed} layer of water molecules around the addition complex.

Step 3) can be eliminated as a possible rate-limiting step for association, since the isoequilibrium relationship would not be expected to hold for such a wide variety of ligand structures as we have studied. Differences in the physical interaction mechanisms of n-alkyl, cycloalkyl, 1-3-dioxolane derivatives as well as ligands bearing ester functions with the protein molecule, would without doubt introduce significant deviations from the isoequilibrium relationship. Were it be so, the extrathermodynamic relationships for separate groups of ligands (such as those assembled in Table V) would have slopes and intercepts differing markedly from each other. As was shown above however, this is not the case.

One would normally suspect that the association step for the "naked" partners should be diffusion-controlled: strong supporting evidence for this contention may be found in the rate of formation of inclusion compounds (which is a good model system for enzyme-substrate inter-

actions). Cramer (140) could recently show that the "...rate of recombination of the complex formation (with α -cyclodextrin) of nitrophenol and its anion... are almost of the order of diffusion-controlled reactions..." Furthermore, chemical interactions in general are very fast processes; for example, the formation of hydrogen bonds between partners is diffusion-controlled as the rate constants are of the order of 10^{-10} - 10^{-11} sec^{-1} (141). Therefore, the actual binding step (step 3) must be ruled out as a possible rate-limiting step.

We therefore have concrete evidence here that the rate-controlling step for the binding process concerns external physical factors, the most important of which being the structure-making or structure breaking effects of the various ligands on the surface-bound water.

Whether step 1 or 2 is determinant has no bearing on the present discussion. It is only necessary to draw attention to the marked effects on binding of the stereochemical features of the TMA derivatives of the 1-3-dioxolane series to be convinced that the highly specific protein surface is implicated in the observed effects on the water structure.

As a working hypothesis, we could state that the controlling parameter of complex formation is the effect of the ligand structure on the arrangement of the water molecules at the binding site. The equilibrium constants exhibit a temperature dependence which is not related to

the strength of the interaction, but to the physical arrangement of water molecules.

C) Models of Ligand Interactions:

We wish to report here two observations taken outside the field of purely biological systems, that give support and confer likelihood to the above interpretation. The first one concerns the study by Cramer (140) of the thermodynamics and kinetics of formation of inclusion compounds with α -cyclodextrins. The conclusion reached by the author who applied relaxation techniques, is that the rate-limiting step for the formation of such inclusion compounds consists in the melting of a few water molecules surrounding the azo dye ligand which is to enter the cavity supplied by the cyclodextrin ring.

Since Cramer reports the equilibrium constants as well as their temperature dependence for a series of ten azo dyes forming inclusion compounds, it was of interest to analyze his data with a view to verifying the possible operation of an isoequilibrium relationship and in the eventuality of an affirmative answer, establish the magnitude of the slope β . The ΔF quantities for the binding process have therefore been calculated using the K_{eq} values given by Cramer and the entropy terms calculated in the usual manner, as discussed in Chapter I, (page 43). The relevant data thus obtained are assembled in Table VIII.

TABLE VIII

Thermodynamic parameters for the formation of inclusion
compounds between α -cyclodextrins, and a series of azo dyes

Name of the Substrate	Compound N ^o	Keq. ^{a)b)} $\times 10^{-3}$	ΔF 14 ^o Kcal./mole	ΔH ^{b)} Kcal./mole	ΔS E.U.
nitrophenol	1	2.6	-3.4	-4.2	- 2.8
nitrophenolate	2	0.27	-4.7	-7.2	- 8.7
1-hydroxylate-4-(4'-nitro)phenyl azo-8-naphthalene sulphonate	3	3.2	-3.3	-6.3	-10.4
4'-hydroxyphenyl azo-1-naphthalene-4-sulphonate	4	3.7	-3.3	-7.0	-13.2
4'-hydroxylatephenyl azo-1-naphthalene-4-sulphonate	5	1.55	-3.7	-6.3	- 9.1
4-dimethyl aminophenyl azo-1-naphthalene-4-sulphonate	6	0.99	-3.9	-7.1	-11.1
3'-methyl-4'-hydroxyphenyl azo-1-naphthalene-4-sulphonate	7	2.4	-3.4	-6.4	-10.4
3'-methyl-4'-hydroxylatephenyl azo-1-naphthalene-4-sulphonate	8	2.1	-3.5	-5.8	- 8.0
3'-ethyl-4'-hydroxyphenyl azo-1-naphthalene-4-sulphonate	9	2.2	-3.5	-6.5	-10.4
3'-ethyl-4'-hydroxylatephenyl azo-1-naphthalene-4-sulphonate	10	3.5	-3.2	-7.7	-15.7

a) Keq = the equilibrium constant for inclusion compound formation

b) values as reported by Cramer (140)

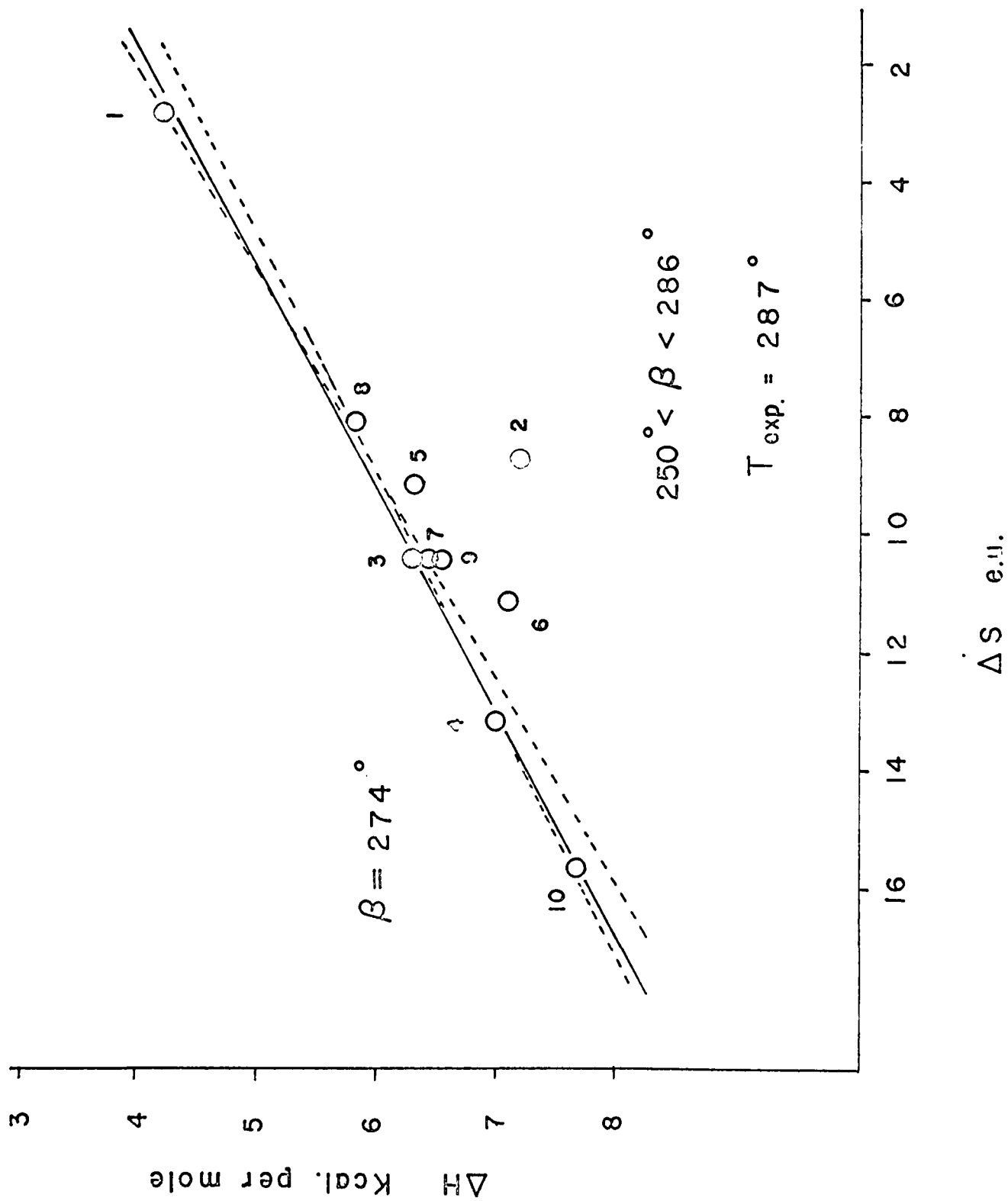
From these data, an isoequilibrium plot was constructed and is shown in Figure VIII (page 87). It can be readily seen that the spread in the ΔH and ΔS terms is not as large as in our case; but this is immaterial since we are not concerned here with the establishment of a unique mechanism but rather with the magnitude of β by comparison with the slope of the curve shown in Figure 6 (page 62). A graphical and rough evaluation of the slope of the curve on Figure 8, gives a value for β which lies between 250° and 286° . This is strongly suggestive of a mechanism where the structure breaking effect of a ligand molecule on bound water determines the size of the thermodynamic parameters, a conclusion arrived at by way of a completely different approach, namely the relaxation techniques; furthermore, it will be noted that the value of β for the isoequilibrium relationship describing the mechanism is close to the average experimental temperature (287°). Since the value of β is of the same order of magnitude as the value obtained for our ligand-enzyme system, our proposal that the removal of water molecules from the surface of the reacting species is the rate-limiting step for complex formation becomes much more acceptable as a valid approximation of reality.

The thermodynamics of addition complex formation between polyvinyl pyrrolidone and aromatic compounds has been investigated by Molyneux and Frank (139). The evidence also points to a determinant effect of the ligand on the structure of water around the cosolutes. An isoequilibrium

Figure 8

Isoequilibrium curve for the formation of inclusion
compounds with α -cyclodextrin

(The thermodynamic parameters were obtained from
Cramer's report (140)).



plot characteristic of that system (ref. 139, Figure 5) was constructed and a slope β of 340° was estimated. This has been interpreted by the authors as an indication that the temperature dependence of the equilibria for complex formation is under the control of structure-breaking effects between water and the polymer surface.

Other examples involving simpler systems taken from the field of the chemistry of metal complexes also support the view that the rate-limiting step for the formation of addition complexes is the loss of a water molecule from the inner hydration sphere of certain metal ions (142). This conclusion was confirmed using data obtained by relaxation methods, a subject which was exhaustively reviewed by Eigen and de Maeyer (143). It was shown that the rate constant for the substitution of one anion for one water molecule is consistently of 10^9 sec^{-1} , a value which is not specific to the metal ion, whereas the rate for the removal of one water molecule is specific to the metal and is much lower by a few orders of magnitude.

The above facts support the conclusion that the rate-determining step for complex formation between AChE and TMA ligands ought to be the removal of structured water molecules from the area of the binding sites.

D) Nature and Role of Protein Bound Water:

No special hypothesis is required in order to anticipate the involvement of structurally bound water on protein surfaces. It has long

been known that protein molecules are not only loosely hydrated as are most organic molecules in aqueous solutions, but actually include a real water lattice, the "ice-like" structure of which being expectable on the basis of the iceberg concept introduced many years ago by Frank and Evans (144). Consequently, some aspects of protein behavior, as revealed for instance by titration curves or denaturation processes, could be clarified when the fact was recognized that a highly structured network of water molecules forms an integral part of the molecule (145). In addition, as was pointed out above, recent dielectric measurements have shown that the structure of this protein bound water is "ice-like" in character (137). It would be appropriate here to postulate the existence of another type still of bound water which would specifically make part of the three-dimensional protein structure. Such a possibility would arise from the presence on the protein surface and especially at the active center of some key polar groups so conveniently located with regard to the environment, that they would specifically accommodate a molecule of water. As an example of such a special type of specifically bound water, one may cite the case of the water molecule which is believed to occupy the sixth coordination position of the iron atom in the deoxygenated hemoglobin molecule (155,156).

Current evidence pertaining to the tertiary structure of the enzymes α -chymotrypsin (146) and ribonuclease (147), indicates that the active center lies in a cleft or a depression on the surface. This region

of the molecule may well possess special features which could favor specific interactions with a few water molecules; these would then not make part of the structural network around the macromolecule and would have chance to play a rather more specific role. It is obvious however, that the thermodynamic approach to the problem of water extrusion cannot allow a distinction between these two fundamental types of highly structured water associated with the protein.

Other physico-chemical parameters will have to be discovered in order to locate any strategic position that bound water molecules may occupy. In this regard, it is of interest as well as suggestive that some of the ligands studied in this work strongly influence the parameter of pharmacological activity as reflected in their acetylcholine-like actions. It is not inconceivable that their interaction mechanisms with AChE may profitably be compared with their effects on the ACh-receptors since the latter are highly sensitive to the stereo-electronic properties of the ligands. This need for an additional parameter of interaction specificities stresses the limitations of the thermodynamic approach with regard to the problem of biological specificity. In other words, what is important to know is the consequence of ligand binding with special reference to a certain biological response, and not exclusively the total number of water molecules extruded by the ligand from the cleft.

Accordingly, an approximate definition of protein specificity toward ligands would be as follows: the ligand binding must be considered in relation to the strategically bound water molecules which when removed from the cleft lead to a significant modification of the protein conformation. Hence, enzyme specificity is intimately related to the stabilization of one favorable protein conformation over many others; it can be seen that strength of binding for a ligand bears no relationship to the basic problem under consideration.

E) Transitions in Protein Conformations:

The bindings of ligands on the protein surface being accompanied by the removal of strategically bound water molecules may possibly lead to important reorganization of the protein structure, such as the making or breaking of new intramolecular hydrogen bonds, the aggregation or dissociation of subunits, or helix-coil transitions within the polypeptide chain. We have seen above that intramolecular H-bond formation can hardly be the rate limiting step for such changes. In addition, the characteristic value of 288° for β , the slope of the isoequilibrium relationship of Figure 6, supports the view that even though transitions in protein conformations may arise subsequently to the binding, they could not be rate-limiting because they appear not to be reflected in the ΔH and ΔS parameters. Were it be so, the value of the slope would be expected

to be significantly different from the temperature of melting of ice. Furthermore, the algebraic sum of the bond breaking and bond forming effects accompanying any transition could hardly be expected to leave ΔF constant and at the same time permit exact compensation effects between the ΔS and ΔH quantities.

Hence, it is quite probable that important conformational changes occur upon complex formation with the ligands and this in spite of the fact that water extrusion is the only rate-limiting step, that is thermodynamically observable. If the conformational changes were to be directly evaluated, other physical or chemical methods would have to be used. This way of considering the binding specificity of substrates and inhibitors in terms of the stabilization of protein conformations is of course in complete agreement with the known adaptability and flexibility of many enzyme molecules.

To illustrate better the manner in which water molecules attached to protein can stabilize their tertiary and quaternary structures, it seems appropriate to refer to the model recently proposed by Warner (148) so as to account for the mechanism of the polymerization of the tobacco mosaic virus (TMV) molecule. According to this model, the polypeptide chain of TMV would be rolled-up in a spiral-like fashion. In the coiled molecule, the peptide carbonyl oxygens would form an hexagonal arrangement which is consistently preserved as the diameter of the rolled

chain gradually increases. Such an arrangement provides for a hydrophilic surface alongside the spiral where the carbonyl oxygens are laid down. A hydrophobic surface would lie on the opposite side and would consist of the amino acid residues. Molecular models have been also constructed for the simpler hexapeptide ring of the insulin A-chain, for the decapeptide gramicidin S, and for the insulin B-chain (M.W. 3,000). Warner has proposed for the case of the TMV molecule that three rolled chains would combine in a radial fashion so as to form the basic subunit of the macromolecule. One can readily see that the hydrophilic surface can interact through H-bonding with water molecules. The hexagonal pattern for the carbonyl oxygens would even favor the formation of "ice-like water" all over the hydrophilic surface; in fact, there is a striking resemblance between the pattern of the hexagonally disposed oxygens and the characteristic pattern assumed by the oxygen atoms in ice. The oxygen-oxygen distances in both patterns are almost identical.

On the basis of such observations, Warner has proposed that the hexagonal pattern of the polypeptide chains is stabilized by the formation on the hydrophilic surface of an "ice-like" network of water molecules; the formation of the "ice-like" structure is of course mutually facilitated and stabilized by the arrangement of the hexagonal carbonyl oxygens. Thus, we finally arrive at a satisfactory picture of the TMV molecular unit: it would consist of stacked subunits as a result of the mutual interactions between the hydrophilic and hydrophobic surfaces of the subunits. A layer of water molecules would be responsible for subunit stabilization and would be "sandwiched" somehow between the subunit outer surfaces.

Note: According to Warner, the TMV molecule envisaged as a polymer would possess a rod-like shape with an hexagonal section. Such a model of protein conformation may be of general significance and may apply to the AChE molecule. In fact, Leuzinger and Baker (138) recently crystallized the latter enzyme (from E. electricus) and showed that the crystals assume a hexagonal-rod shape.

It is beyond the scope of this thesis to evaluate the absolute validity of Warner's model. What is really important to us is that it provides a rational basis for the interpretation of the effects of water extrusion on the conformational stability of the protein. The really important feature of the model is the geometrical arrangement of the carbonyl oxygens which assume a fixed hexagonal structure where the oxygen-oxygen distance would amount to about 4.8 Å. One would readily expect that the binding of a ligand within a specific area of the spiral could well disrupt or rather interfere with the formation of the required hexagonal ring; this would favor the unrolled conformation for the polypeptide and a shape other than the hexagonal one would now be stabilized by the ligand. Consequently, the formation of the stabilizing water layer over the hydrophilic surface would be impaired, at least at the sites where the carbonyl oxygen pattern has been destroyed.

Other mechanisms are possible through which ligand binding may affect the stability of the protein conformation. For instance, the hexa-

gonal geometry could be preserved but the ligand only affects the outer layer of water molecules covering the hydrophilic surface. The local "damage" would here be much smaller but it could certainly be significant enough to prevent for example a precise stacking of the subunits. In other words, a ligand might exert a weak local effect but could nevertheless impair the aggregation of subunits or directly prevent the coiling of a polypeptide chain.

In the hypothetical case of the binding of a ligand onto hexagonally arranged polypeptide segments, the only detectable thermodynamic changes will thus be the rate-limiting expulsion of a certain number of water molecules from the surface; any disruption of the subunit-subunit bonds, as in the case of the TMV molecule, would most likely not be detected by our method of approach. (The presence on the subunit surface of some dehydrated areas will contribute to a decrease in the surface of contact between hexagonal segments on adjacent subunits, so that an interaction (say H-bonding) that would have normally occurred between two subunits through the "ice-like" layer will not occur under such conditions).

This model serves to anticipate some of the fundamental changes in the overall physical properties of protein molecules, that may possibly take place as the result of water extrusion, as caused by the binding of a ligand. It also provides a rational basis for the hypothesis that the presence of orderly oriented water molecules can form an integral part of the tertiary and quaternary structure of enzymes.

F) Water in Relation to Receptor Behaviour:

The water extrusion hypothesis may be profitably analyzed in relation to its possible implications at the ACh-receptor level. There exists no evidence that AChE and the ACh-receptor possess identical structures, so that the direct extrapolation of our results to the physiological receptor structure is not permissible at this stage. However, one should certainly consider the applicability of the concept of water extrusion, as it may provide the pertinent parameter allowing predictions about the potential ability of cholinergic drugs to mimic the effects of the normal substrate ACh. In this regard, the following facts will have to be borne in mind: recent findings on the structural properties of membranes (3), in relation to active transport phenomena, suggest that the permeability of the cell structure may be drastically affected by slight changes in the conformation of the proteins that make part of the membrane (80).

A model was recently proposed by Hechter (149) which purports to account for the distribution of sodium and potassium ions across membranes. An important aspect of this model is the proposal according to which the water present in the "channels" of the membrane would have an "ice-like" structure. The author thus writes: "...The essence of the proposal is that polarized membrane is envisaged as a precisely ordered lattice involving arrangements of monolayers of protein, lipids and "ice-like"

water, depolarization as the induction of localized "disorder", each component exhibiting a characteristic phase transition which then spreads through the membrane phase". (149, p. 637).

The water extrusion hypothesis represents a specific refinement of this crude model: the removal of water molecules present as normal constituents of the membrane, would be sufficient to induce the postulated "disorder", which triggers a "transition" that is eventually reflected in the observed stimulus.

In our view, it is of prime importance that the water molecules to be extruded, should occupy strategic positions in the receptor membrane. In fact, a disruption of the water layer that may be viewed as not forming a structural part of the membrane receptors, would most probably not induce the desired transition leading to a stimulus. However, the water molecules that would be situated in the strategic "channels" of the membrane (as in Hechter's model) would induce a significant transition, if extruded or disordered. This type of water is what we refer to as "strategically bound water". Our proposed physical interaction mechanism for which we have actual model experimental evidence (in contrast to Hechter) may well provide a rational basis for the interpretation of receptor specificity toward various types of cholinergic drugs.

G) The Nature of "Strategically Bound Water Molecules":

An important step toward the establishment of a more complete and clearer understanding of the overall processes underlying cholinergic activity would consist in producing direct evidence that a conformational change actually occurs upon binding of an activator ligand. Such evidence is not available at the present time. On the other hand, the water extrusion hypothesis for which evidence exists offers the possibility of relating pharmacological activity to ligand-induced conformational changes in the receptors. The anti-ACh activity of a ligand upon binding to the AChE surface may be explained on a similar basis. The conformational perturbation theory (77) predicts that there should exist an equilibrium between two conformations for AChE and the cholinergic receptors. Substrates and other agonists would modify the conformational equilibrium as they bind to the respective surfaces of the enzymes and the receptor. Antagonists would stabilize another ineffective conformation for the receptor.

It then becomes easier to define what is really meant by "strategically bound water". For the case of our AChE-ligand system, it can be safely postulated that ACh being the natural substrate, would effectively stabilize one specific and unique conformation for the enzyme. This perturbation would primarily be the result of water extrusion from the active sites themselves; this water must be strategically bound since it

occurs at the chemically significant region of the protein. As a result of this extrusion, the original conformation would lose its stability and the protein would undergo some reorganization to a more stable form. Other ligands could extrude as many water molecules but from less strategic sites so that they would be unable to destabilize the original conformation to the same extent as does ACh. It may be recalled that we are here dealing with an equilibrium situation so that a complete spectrum of stabilizing activities with respect to the effective perturbation will exist.

As pointed out above, however, the thermodynamic approach does not allow a definitive conclusion as to whether the conformational change takes place. On the other hand, the pharmacological activity of the ligands at the ACh-receptor level provides an additional means of testing the predictive value of the water extrusion hypothesis as encompassed by the general perturbation theory of receptors. More specifically one may be in a position to decide, on the basis of agonistic potencies, whether a ligand may extrude strategically bound water or not at the AChE level. It is clear that if the receptor surface suffers the hypothetical "normal" conformational change (77), as the result of a removal of strategically bound water molecules, the binding of the drug will be conducive to a strong physiological stimulus. In that sense, the pharmacological activity of a certain ligand may be regarded as confirming that it may or

may not possess the right structural features allowing it to mimic the stabilizing effect of ACh on the effective conformation.

The water extrusion hypothesis establishes a link between the general conformational perturbation theory of drug action (77) and the key role of protein-bound water. Although definitive evidence that a conformational change occurs, is still lacking, Changeux (80) has recently proposed that in the case of AChE (from *Torpedo Marmorata*), there would exist an equilibrium between two different conformation states of the enzyme: this view served to account for certain unusual kinetic properties of the enzyme. According to Changeux the conformational change that would take place in the protein would be transmitted to the membrane and would thus affect the state of polymerization of the receptor.

In the light of our mechanism studies on water extrusion and of generally held views on the conformational properties of proteins and other receptors, it is worthwhile taking a second look at the theoretical basis of interaction specificities.

H) The Meaning of Enzyme Specificity:

It is now clear, from what has been said above, that a specific enzyme-ligand interaction must first be defined in terms of specific dehydration effects before anything can be decided about the nature of the interaction forces at play. Strong binding can be largely controlled

by the removal of a large number of "ice-like" water molecules from the protein. Since this represents an unspecific type of binding, the often assumed direct relationship between intensity and specificity of the ligand interaction must be dismissed as invalid on the basis of the above considerations.

Also, we recognize that it is not possible to obtain a direct and definitive proof for specific binding onto an enzyme, using the thermodynamic approach as the only basis. However, and as was pointed out above, if the size of the thermodynamic parameters for the binding of certain ligands is anomalous relative to the effects of a series of reference ligands, the conclusion is permissible that a specificity effect is operative. It is probable that the use of complementary experimental methods such as O.R.D. or the use of reporter groups (130), could serve to demonstrate the occurrence of specific conformational changes in the protein. A combination of such methods with the thermodynamic approach would be amply sufficient to define a given interaction in terms of specific or unspecific interactions.

It is of interest in this connection that an evaluation of the pharmacological activities of cholinomimetics may in fact provide us with the desired complementary parameter that is necessary to confirm the occurrence of true specificity effects. Indeed, a ligand that would specifically interact with the ACh-receptor should be one that is capable of stabilizing maximally the effective conformation of the receptor, in a manner paralleling

the effects of the natural ligand ACh. The cholinergic activity of a number of key ligands described in Table I will be discussed later; this biological data shall be used in conjunction with the thermodynamic parameters for binding on AChE in an effort to illustrate the general significance of interaction specificities.

I) Non-Specific Binding:

a) TMA-cycloalkyl derivatives:

Examples of non-specific binding are found in the series of the cycloalkyl-TMA derivatives. These molecules, which bear little analogy to ACh ought to induce, upon binding, the melting of the "non-specifically bound" water on the enzyme surface. In this series, the enlargement of the ring by one methylene group causes a simultaneous change in two parameters, namely the molar volume and the rigidity of the ring. Because of the inherently limited number of ring sizes in the series, it is not possible to determine exactly whether molar volume or ring rigidity (or both) controls the thermodynamic parameters. However, and as expected, an enlargement of the hydrocarbon ring is reflected, upon binding on the enzyme, in the destruction of an increased number of structured water molecules, since both the enthalpy and entropy quantities assume more positive values. This phenomenon is clearly illustrated by

the respective behaviour of the cyclobutyl, cyclopentyl, and cyclohexyl rings where gradual increases in volume are neatly paralleled by increases in the ΔH and ΔS values.

The seemingly anomalous behaviour of the cycloheptyl- and cyclooctyl-TMA derivatives is explained by the much increased flexibility of these large rings which, by comparison with the smaller rings, possess many more internal degrees of freedom. This explains their "open-chain" like behaviour on the enzyme surface (Tables IV and VII, pp. 57,71). Despite a theoretically favorable entropy originating from the melting of "ice-like" water, much energy would be needed in order to restrict the internal motions of the larger ring ligands; this latter entropy demand cancels the favorable terms, with the result that these cyclic TMA derivatives are thermodynamically almost indistinguishable from their open-chain relatives.

This statement finds additional support in the fact that for compounds possessing identical volumes such as the bicycloheptyl and the bicyclooctyl derivatives where rigidity is actually maintained in the rings, the expected net gain in entropy is observed which means that an increased extrusion of water molecules occurs. It is worthwhile noting that when comparing the bicycloheptyl and bicyclooctyl compounds with the cyclohexyl ring, it is observed that the extrusion of water molecules is not in proportion with the increase in molar volume. This can be accounted

for by the fact that binding is a surface phenomenon and that the six-membered ring is the feature that enters into contact with the surface of the protein (the bridged methylene groups would not extrude water molecules from the surface because no contact would be achieved).

The binding of the adamantyl ring would be expected, on the basis of the preceding considerations to exhibit thermodynamic quantities at least as large as those that characterize the binding of the bicycloheptyl and bicyclooctyl ligands. The finding that the ΔH and ΔS values are in fact significantly lower than expected, points towards a special effect induced by this symmetrical cage compound. It is reasonable to postulate that the highly symmetrical arrangement of the atoms in this molecule may induce a special organization of the water structure around the rings, such as the formation of a clathrate-like arrangement. Such an effect would make ΔH and ΔS more negative than expected.

The non-substrate ester ligands, such as D(-)- β -methyl ACh (compound No 21) and "reversed" ACh (compound No 22), are characterized by relatively small H and S quantities. They exhibit thermodynamic parameters similar to the open-chain analogs n-butyl- and n-pentyl-TMA (see Table VII), thus establishing that these ester structures interact rather non-specifically.

b) aromatic ligands:

Within a single series of related ligands, it is useful, as was done above, to establish comparisons between individual members. But the basis for comparing the effects of ring size on the thermodynamic parameters for compounds belonging to two different series is somewhat unsafe. Nevertheless, we can see in Figure 6 that two chemically distinct six-membered ring compounds (cyclohexyl- and phenyl-TMA, compounds No 3 and No 12) possess comparable ΔH and ΔS values, thus providing a basis for establishing the relative importance of aromatic character with regard to thermodynamic behaviour. However, the same conclusion does not apply to the ligand pair furan-cyclopentyl; in fact the furane derivative (compound No 9) is thermodynamically not equivalent to the cyclopentyl analog, the former exhibiting significantly more positive ΔH and ΔS values. The discrepancy may be attributed to the presence in furan of an oxygen atom, a feature which might favor the release of more water molecules than expected.

One will readily note that the increase in molar volume attending the addition of one methyl group to the furane ring (compound No 9) results in an actual decrease (1 Kcal.) in the enthalpy of binding, an effect entirely opposite to the established trends since the extra methyl group ought to cause the displacement of more water molecules. This result may be explained like the adamantyl case, by postulating a net

structure-making effect by the methyl group such as would be the situation if a partial clathrate cage would form around the methyl group.

Since only four aromatic ligands have been investigated, it is not possible to determine with precision the net effect of aromatic character or of the presence of heteroatoms therefrom on the extrusion of water molecules from the enzyme surface. Nevertheless, one can easily see that this parameter of water displacement is the one that is really sensitive to specificity effects and not the parameters of H-bonding between heteroatoms and the enzyme or of charge-transfer interactions since they are not rate-limiting.

c) isosteric changes:

A comparison between the 2-furyl- and 2-thienyl-TMA derivatives (compounds Nos 9 and 11) indicates that the isosteric change of an oxygen for a sulfur atom leads to a considerable and unexpected effect on the thermodynamics of binding. The difference of 5 Kcal. in $\Delta H_{\text{binding}}$ between the two must remain unexplained presently; this finding may explain the frequently observed non-equivalence of furan and thienyl rings in drugs affecting physiological receptors. This type of chemical change has often been regarded as insignificant by the pharmaceutical chemist. An example of the unexpected sensitivity of biological receptors to the

nature of the heteroatom is found in the respective activities of biotin and oxybiotin. The sulfur-containing molecule possesses much higher activity than the oxygen analog. As far as is known, no satisfactory explanation, at the molecular level, has been given for these differences. It would seem, on the basis of our results, that the enzyme surface can differentiate between these two isosteres just as readily as the olfactory receptors can; the replacement of a drug by an isostere may often lead to drastic differences in the number of water molecules actually extruded. This would of course not be reflected in the relative affinities of the analogous ligands.

d) The effects of configuration

The release of water molecules from the enzyme surface can be quite sensitive to configurational changes introduced in the ligand molecules. The thermodynamic parameters that characterize the binding of the D(-)- and L(+)-1,3-dioxolane-4-methyltrimethyl ammonium iodide to AChE serve to illustrate this point. In addition, when compared with these latter compounds (Nos 13 and 14), the isomeric 1,3-dioxolane-2-methyltrimethyl ammonium iodide (compound No 19) produces very characteristic effects.

While the introduction of a ring methyl group in the L-series of 1,3-dioxolane ligands produces very important fluctuations in the thermodynamic parameters of binding, the same substitution in the enantiomeric D-series is without any effect. As was mentioned earlier, the binding of all the dioxolane derivatives of the D-series appears to be thermodynamically equivalent. In all these cases, the binding parameters can be accounted for by their molar volumes and their relative flexibilities, and in this respect they behave like the cyclopentyl ligand. These ligands of the D-series would bind on a region of the enzyme surface (with the attending consequence of non-specific bound water extrusion) where the ring methyl group, irrespective of its configuration, would be unable to stereospecifically extrude bound water molecules, because of the unfavorable D-configuration of the ligands.

On the other hand, the ligands of the L-series would occupy a somewhat different area on the surface, where specific extrusion of water (as evidenced by the case of the L-cis derivative, compound No 15) would occur. This possibility will be discussed later. If we assume that the L(+)-1,3-dioxolane-TMA derivative (compound No 13) would interact with a specific area, it might be expected that introducing a ring methyl group in the 2-trans position will now be unfavorable to binding on the specific area; this allows the prediction that the L-trans derivative (compound No 17) will chance to bind on the area where the D-enantiomers interact. This view is supported by the fact that the thermodynamic parameters for the L-trans compound (Table IV, p. 58) are comparable to those for the D-series of dioxolanes (compound Nos 14, 16, 17, 18).

One may then conclude that, while the enzyme surface may be sensitive to the configuration about a single asymmetric center, it may remain relatively insensitive to the configuration about a second asymmetric center as long as the latter does not assume a specific orientation such that the methyl substituent can play a unique role in extruding water molecules.

Note should be taken here that clathrate formation is a definite possibility in the case of certain ligands in the 1,3-dioxolane series. Such a formation would affect the value of the thermodynamic parameters of binding. The recent findings of D.W. Davidson (154) on the formation of

the clathrate 1,3-dioxolane.17H₂O supports the expectation that the structure of the hydration water for these compounds may be strongly influenced by the steric disposition of a ring substituent. The binding of such ligands on the AChE surface could result in the destruction of the clathrate structure in the case of one isomer but could be without effect in the case of another isomer. Such effects would be largely reflected in the thermodynamic parameters.

J) Specific Binding:

Since ACh is the normal substrate for AChE, it ought to interact specifically with the enzyme. It was seen above that the non-substrate esters D(-)- β -methyl acetylcholine and "reversed" ACh exhibit thermodynamic parameters which are in accord with their chain-like structure, and this in spite of the presence of an ester function in their molecules. The thermodynamic parameters for ACh are quite unusual in the sense that they are indicative of a considerable efficiency of the molecule in extruding water molecules; these water molecules can only be bound at strategic sites since the molecule is rapidly hydrolyzed by the enzyme. According to our interpretations, the ACh is able to release a large number of specifically bound water molecules from the surface, a capacity which is not shared by the non-substrate esters.

The rapid hydrolysis of ACh by the enzyme would then be consequent to its special ability to remove strategically bound water molecules in correct number. That this is the case is evidenced by the fact that another ester compound, the L(+)- β -methyl acetylcholine, which is also a substrate, is characterized by ΔH and ΔS values that are of the same order of magnitude as the values for ACh. It follows that the L(+)- β -methyl ACh molecule would also induce the necessary release of strategically bound water molecules in a way similar to ACh: this observation correlates with the fact that it is a substrate for AChE in contrast to its D-enantiomer. In this connection, the fact that D(-)- β -methyl ACh is not a substrate does not necessarily mean that there is steric interference to a close approach of the esteratic site by the ester function; it is possible that the unfavourable position of the β -methyl group would preclude an adequate dehydration of the catalytic sites.

A similar explanation would serve to account for the fact that 3-acetoxyquinuclidinium methyl iodide is a substrate for AChE (37), while the isomeric 1-(acetoxyethyl) quinuclidinium iodide acts as an inhibitor (38), and this despite the fact that the ammonium head and the ester group are separated in both compounds by an identical number of atoms.

With regard to induced conformational changes, the above results with substrate-ligands would indicate that hydrolysis is possible only when a specific physical state of the protein is initially stabilized (80) by the proper removal of a certain number of strategically bound water molecules.

The L-cis-2-methyl-1,3-dioxolane-4-methyltrimethyl ammonium derivative (compound No 15) would also be effective in maximally stabilizing the same specific physical state that would otherwise be conducive to hydrolysis, if the ligand were susceptible to attack by the nucleophiles. The introduction of a methyl group in the 2-cis position of the L(+)-1,3-dioxolane molecule (compound No 13) increases $\Delta H_{\text{binding}}$ by about 5 Kcal. and ΔS by ~ 19 e.u. These large increases are to be compared with those that characterize the change-"reversed" acetylcholine \rightarrow acetylcholine ($\delta \Delta H = 4.6$ Kcal., $\delta \Delta S = 20$ e.u.) and the change D(-)- β -methyl ACh \rightarrow L(+)- β -methyl ACh ($\delta \Delta H = 6.7$ Kcal., $\delta \Delta S = 23$ e.u.). Hence, either the stereospecific addition of a methyl group, or an inversion of configuration or a transportation of the oxygen atom in ACh may be said to produce highly specific effects on the enzyme. Accordingly, the specific extrusion of water molecules is reflected in the relative magnitudes of the thermodynamic parameters which in the case of the substrate ligands, correlate with rapid hydrolysis.

Although the L-cis-1,3-dioxolane derivative would maximally stabilize the proper conformational arrangement in the enzyme molecule, the actual perturbation cannot be detected kinetically since no hydrolysis occurs. A useful parameter which may allow conclusions regarding the question as to whether the L-cis ligand stabilizes the same physical state as ACh, would consist in its relative ability to stimulate the ACh-receptors.

K) Pharmacological Activity:

The muscarinic potencies, as supplied by Dr. A. Friesen*, are given in Table IX (page 114) for ligands of the 1,3-dioxolane series as well as for other physiologically active molecules. The eserinated guinea-pig ileum was used and the potencies were calculated from complete dose-response curves. These data clearly reveal the unique position occupied by the L-cis-dioxolane derivative relative to the 1,3-dioxolane analogs as well as all the other ligands tested. The muscarinic potency of this ligand is at least twice as large as the activity of ACh, thus confirming the expectation that it strongly stabilizes the receptor conformation that is also stabilized by ACh itself. The addition of a cis-methyl group to the L(+)-isomer of 1,3-dioxolane-4-TMA (compound 13), increases the activity of this analog by a factor of 100; a similar spe-

* Department of Pharmacology, Faculty of Medicine, University of Ottawa.

TABLE IX

Relative Pharmacological Activities of a Few TMA-Ligands

(ACh being given the arbitrary activity of 1)

Compound No.	Name	Activity ^{a)}
23	acetylcholine	1
20	L(+)- β -methylacetylcholine	1
21	D(-)- β -methylacetylcholine	0
13	L(+)-1,3-dioxolane-4-methyltrimethyl ammonium iodide	0.02
14	D(-)-1,3-dioxolane-4-methyltrimethyl ammonium iodide	0
15	L-cis-2-methyl-1,3-dioxolane-4-methyl-trimethyl ammonium iodide	2
16	L-trans-2-methyl-1,3-dioxolane-4-methyltrimethyl ammonium iodide	0.2
17	D-cis-2-methyl-1,3-dioxolane-4-methyl-trimethyl ammonium iodide	0
18	D-trans-2-methyl-1,3-dioxolane-4-methyltrimethyl ammonium iodide	0.1
9	2-furyl-methyltrimethylammonium iodide	0.05
10	5-methyl-2-furyl-methyltrimethyl-ammonium iodide	0.4
2	cyclopentyl-methyltrimethylammonium iodide	0.01

a) the activity is given as a fraction of the activity of ACh.

cificity effect is operative at the level of the thermodynamics of binding onto AChE. The pharmacological activities are in complete agreement with expectations based on the thermodynamic parameters for binding. Those ligands that we deduced to act as non-specific releasers of bound water molecules, consistently exhibit very low pharmacological activities. This is the case, for example, of the cyclopentyl-TMA compound as well as the 1,3-dioxolane derivatives of the D-configuration. On the other hand, certain ligands, namely ACh, L(+)- β -methyl-acetylcholine and the L-cis-1,3-dioxolane derivative, that were deduced to extrude from AChE "strategically bound water molecules" (thus initiating the conformational stabilization produced by ACh) possess a characteristically high activity.

The very high specificity exhibited by the L-cis-dioxolane, suffices presently to accept the water extrusion model as a good approximation of reality. The "yes or no" response of the enzyme or the receptor to the ligand, a phenomenon largely conditioned by the presence or absence of the key methyl group is an adequate example of the critical role ascribed to the so-called "strategically bound water molecules". The strategic character of the interaction of the L-cis methyl group is best confirmed by the outstanding muscarinic activity associated with the presence of this seemingly inconsequential substituent.

L) Specificity of the Interactions:

The release of water molecules from the protein surface may now be regarded as the parameter that is actually sensitive to interaction specificities with AChE. Since there presumably exist certain sites that are strategically situated on the enzyme surface, we are justified in proposing that these sites constitute the key sensitive positions that must interact with atoms or groups of atoms in order that the effective conformation conducive, say to hydrolysis of ACh, may be stabilized. A specific interaction with AChE must then always be defined using as the standard of reference the actual physical state normally stabilized by the interaction of ACh with these sites. As a working model, these strategic sites may be viewed as forming strong bonds with water molecules. A ligand which binds specifically to AChE, must then possess a structure capable of extruding a certain number of strategically bound water molecules, a process conditioned by the inherent ability of the ligand to interact readily with the specific sites.

Among the group of inhibitors and substrates that have been investigated in this study, very few can qualify as specific ligands. Besides ACh, which, by definition, is specific to AChE, only the L(+)- β -methyl ACh, the L-cis-1,3-dioxolane-4-TMA derivative and possibly the 5-methyl-furyl-2-TMA (mainly on the account of its muscarinic potency) are able to stabilize the proper conformational state in the enzyme and the receptor.

M) Extension of the Water Extrusion Model:

As indicated in Chapter II, page 43, the Michaelis constants for ACh and L(+)- β -methyl-ACh were treated as true equilibrium constants. The apparent thermodynamic parameters that were derived using this assumption as a basis, were found to obey very closely the isoequilibrium relationship (Figure 6, compounds Nos 20 and 23). This observation does not necessarily support the assumption that K_m is a true equilibrium constant, even if we use as an argument that K_m values can correspond to equilibrium constants as in the case of certain regulatory enzymes (150) and the hydrolase α -chymotrypsin (151, 152). Our parameters merely indicate that the binding of the substrate ligands is also controlled by water extrusion as reflected in the ΔH and ΔS values. The classical representation where the carbonyl group of ACh would assume a tetrahedral configuration by way of nucleophilic attack in the Michaelis complex is not consistent with our observations. Should such a complex form initially, the isoequilibrium relationship would certainly not be expected to hold as well as in the case of the non-ester ligands. It is noticeable that in the case of the substrate ligands, the observed ΔF_{app} for binding is the same as for all the non-substrate ligands. Moreover, the calculated ΔH_{app} and ΔS_{app} values cancel out exactly as in the case of the non-substrate compounds. This would be expected only if the controlling parameter for the binding of the substrates is water extrusion.

CONCLUSION

The water extrusion model should not be necessarily restricted only to the case of AChE. We feel that the model will be generally useful in the evaluation of interaction specificities for many biochemical molecules. The immediate conclusions emanating from the data on AChE will no doubt be applicable at least in part to other systems in the field of protein and enzyme chemistry. Our model is believed to shed a new light on the overall problem of interaction mechanisms between small molecules and macromolecules or between small molecules alone. We may refer to the recent data of J.E. Wilson (153) on the flavin-indole interactions as an illustration of the general value of our studies. The binding to flavine mononucleotide of indole derivatives has been explained on the basis of the operation of dispersion forces and charge-transfer interactions. It is interesting, in the light of the water extrusion model to look more specifically at the reported thermodynamic parameters for complex formation between flavine mononucleotide (FMN) and L-tryptophan on the one hand and FMN and 5-hydroxy-L-tryptophan on the other, (Table X, page 119).

The special effect of the hydroxyl group can be interpreted exclusively in terms of the disruption of the water structure surrounding the aromatic molecules. We observe here the same characteristics that we uncovered using the system AChE-TMA ligands, namely the constancy of

TABLE X

Thermodynamic Parameters for Formation
of FMN Complexes

	ΔF Kcal./mole	ΔH Kcal./mole	ΔS e.u.
with L-tryptophan	-2.7	-7.4	-15.8
with 5-OH-L-tryptophan	-2.9	-4.7	-6.0

the ΔF values and the mutual cancellation of the ΔH and ΔS terms. It is of course impossible to directly evaluate the effect of water disordering around the surfaces of the interacting molecules. But the fact that, in this case as well, the interaction parameters seem to be influenced by water melting, supports the idea that the model has general validity and that it is the thermodynamic parameters that are sensitive to interaction specificities. Water extrusion is a physical model which at the molecular level may serve to explain the mechanisms underlying the regulation of biological catalysts.

CLAIMS TO ORIGINAL RESEARCH

1. The inhibition constants at various temperatures have been determined for a series of 21 tetramethylammonium ion derivatives that act as competitive inhibitors of acetylcholinesterase.
2. The enthalpy of binding at equilibrium has also been evaluated for each compound in this series of competitive inhibitors.
3. The Michaelis constant for the L(+)- β -methyl acetylcholine iodide has been determined at five different temperatures.
4. A physical mechanism has been proposed to account for interaction specificities among a series of competitive inhibitors of acetylcholinesterase. These interaction specificities are reflected in an unusual manner in the size of the thermodynamic parameters of binding, as well as in the muscarinic potencies of the ligands. The model which is proposed claims that the explanation of such specificity is of physical origin as follows: a ligand (competitive inhibitor) which binds specifically can extrude from the enzyme surface a number of "strategically bound water molecules"; consequently, a perturbation takes place in the protein molecule, which is characteristic of the perturbation induced by acetylcholine. In the case of the receptor surface, the rearrangement is conducive to a strong physiological stimulus.

This model is particularly new in that in order to explain interaction specificities between proteins and ligands it refers to the very special role played by a third component, namely protein bound water, while previous explanations of binding processes referred exclusively to the partners, protein and ligand.

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