After cooling to 20° the precipitate was filtered off and the filtrate was evaporated *in vacuo*. The residue solidified on standing and was purified as indicated in Table II.

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# Aspects of the Chemical Mechanism of Complex Formation between Acetylcholinesterase and Acetylcholine-Related Compounds[1a,b]

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The mechanism of acetylcholinesterase (AChE) action is briefly reviewed. The stereospecificity of the enzyme and the nature of the binding forces in the enzyme substrate addition complex are discussed. It is recognized that the active surface of AChE is essentially nonpolar in character, a property which allows the operation of hydrophobic forces in the binding of substrates and inhibitors. The positive involvement of van der Waals attractions is shown to be of rare occurrence. The absolute conformation of enzyme-bound acetylcholine (ACh) is deduced through a study of the stereochemistry of the interaction of 1,3-dioxolane analogs (II) with the active surface. This showed that the enzyme displays both absolute and relative stereospecificity towards this series of inhibitors. Optimum affinity is displayed by the L-(+)-*cis*-2-methyl-4-trimethylammoniummethyl-1,3-dioxolane iodide (VI). The mechanism of interaction of the 2-methyl group with the enzyme was elucidated. Structure–activity relationships could be interpreted on the basis of the operation of hydrophobic forces exerted on the 2-substituents and van der Waals attractions in the case of VI. The free energy of binding for the 2-methyl group of the latter was found to be 1.35 kcal., a value which corresponds closely to the one obtained for the free energy of binding of the ester methyl group of ACh. It is concluded that both ACh and VI uniquely form "lock-and-key" type of fits with AChE. Two modes of interactions with AChE are recognized: (a) one of them involves only the operation of hydrophobic forces, thus necessitating an accommodative perturbation of the nonpolar chains on the enzyme; (b) the other calls into play the net contribution of van der Waals forces and is thus conducive to a highly specific perturbation of the protein. On the basis of these considerations, structure–activity relationships at the receptor level are interpreted. The role of the quaternary ion in catalysis by AChE is discussed; the mechanism of binding of quaternary ions is evaluated critically taking into account some recently proposed modifications of the electronic structure of these ions. The hydrophobic nature of such ions is shown to account for their effect on AChE. Finally, the identical stereospecificities of the enzyme and the muscarinic receptor towards the dioxolane series of quaternary salts suggests near identity of these two bioreceptors for ACh.

Over the past 15 years, much has been learned through kinetic studies about the catalytic mechanism of AChE. It is now recognized that the process of hydrolysis of acetylcholine (ACh) involves the formation of an acetyl–enzyme intermediate which undergoes a rate-determining hydrolysis.[2] It is generally agreed also that the enzyme's active surface includes two kinds of sites: esteratic and anionic. Investigations on the influence of pH on catalytic activity has revealed the existence of esteratic groups having p$K$ values of 6.5 and 9.4, and which are involved in both the acetylation and deacetylation steps.[3] These functional groups have been identified tentatively as belonging to an imidazole ring and a tyrosine residue, respectively. The group of p$K$ = 6.5 is apparently prevented from ionizing in the enzyme–substrate addition complex, thus suggesting that it is masked through interaction with the ester function of the substrate.[3] The esteratic site was also shown to be reactive towards phosphoryl, carbamyl, and sulfonyl derivatives which usually bring about profound inhibition of the enzyme.[4] Finally, the remarkable phenomenon of inhibition by excess substrate has been traced to noncompetitive blockade of the deacetylation step by the substrate.[5] Also, it was shown that the acetyl–enzyme intermediate retains the affinity of the free enzyme towards some of the most common competitive inhibitors.[3,5]

The investigations of Wilson and Cabib[2] on the nature of the forces acting in the enzyme–substrate complex have revealed that the interaction of the third methyl group on the nitrogen of ACh with the enzyme is accompanied by a marked decrease in the entropy of the complex, a result suggestive of a profound structural change in the enzyme. In contrast to this third methyl group which does not contribute to affinity for the enzyme, the other two methyl substituents have a marked effect on affinity, each one favoring adsorption by a factor of 7 over the respective desmethyl analogs.[6] The nature of the forces exerted on these methyl substituents have been ascribed to van der Waals attractions.[6,7] This assumption is discussed in

(1) (a) Published as part III of the series "Studies on the Chemical Basis for Cholinomimetic and Cholinolytic Activity." For part II, see B. Belleau and J. Puranen, *J. Med. Chem.*, **6**, 325 (1963). (b) This investigation was supported by the National Research Council of Canada and represents a portion of the thesis submitted by G. Lacasse in partial fulfillment of the requirements for the M.Sc. degree, University of Ottawa.

(2) I. B. Wilson and E. Cabib, *J. Am. Chem. Soc.*, **78**, 202 (1956).

(3) R. M. Krupka and K. J. Laidler, *Trans. Faraday Soc.*, **56**, 1477 (1960).

(4) I. B. Wilson in "The Enzymes," P. S. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press Inc., New York, N. Y., 1960, p. 501.

(5) R. M. Krupka, *Biochemistry*, **2**, 76 (1963).

(6) I. B. Wilson, *J. Biol. Chem.*, **197**, 215 (1952).

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detail below. It is clear that the marked change in entropy referred to above must have an important bearing on the catalytic mechanism. In addition, a marked acceleration of the rate of acetyl transfer to the enzyme was shown by Wilson and Cabib to be accompanied by a large negative entropy of activation.[2] Thus, AChE must undergo important conformational changes when interacting with its substrate ACh, and these changes should require the presence of a quaternary trimethylammonium moiety. On that basis, attempts at defining the structural and configurational features of the enzyme–substrate addition complex must take into account the macromolecular changes conditioning adsorption on the protein.

The aspect of greatest significance in relation to the mechanism of drug action consists in the mode of interaction between ACh and AChE in the addition complex, and this because most of the numerous competitive inhibitors of AChE, that are physiologically active at the receptor level, effectively compete for the same free active sites that normally serve to bind free ACh. It is therefore of interest to study the structure of enzyme-bound ACh since it is this structure which has the greatest bearing on the mechanism of inhibitor actions both at the enzyme and cholinergic receptor level. The classical representation (I) for the addition complex[8] can obviously be of little use in this connection since nothing is revealed about the stereochemistry of the complex and the nature of the forces contributing to its formation. In addition, the carbonyl group of ACh is assumed in this representation to become tetrahedral in the complex, a suggestion which is clearly arbitrary. On the basis of recent deuterium isotope effect studies,[9] it would seem more probable that the carbonyl remains essentially trigonal in the complex. This paper describes the results of investigations which serve to elucidate some of the most important factors conditioning complex formation between AChE, ACh, and competitive quaternary inhibitors.

Two aspects of complex formation require scrutiny: (A) the stereochemistry of enzyme-bound ACh and (B) the nature of the forces contributing to binding of ACh and related inhibitors of AChE.

**(A) Stereochemistry of Enzyme-Bound ACh.**—Because enzymes are highly asymmetric, it will be expected that when ACh is bound, its normal rotational freedom will be canceled and it will thus assume a rigid conformation. Such a frozen conformation will therefore be optically active and the problem consists in defining the absolute conformation of the bound molecule. Evidence that ACh must assume a highly specific conformation when bound comes from the fact that AChE displays high stereospecificity towards the optical forms of the analogous substrate, β-methyl-ACh, only the D-isomer of the latter acting as a substrate for the enzyme.[10] Furthermore, the investigations of Witkop, *et al.*,[11] have revealed that AChE also displays some degree of relative stereospecificity towards the muscarine series of stereoisomers. It is surprising that virtually nothing is known yet about the absolute stereospecificity of the enzyme towards Michaelis complex formation with the quaternary series of inhibitors. It was of interest to learn about this type of specificity, if anything is to be known about the stereochemistry of bound ACh both at the enzyme and cholinergic receptor level.

**(B) Nature of Binding Forces in the ACh–AChE Complex.**—In order that the chemical consequences of complex formation between AChE and quaternary inhibitors may be interpreted, it will be essential to elucidate the nature of the forces involved in complex formation. Four types of forces favoring adsorption onto the active surface may be involved: (1) Coulombic attractions, (2) hydrogen bonding, (3) van der Waals forces, and (4) hydrophobic interactions. The investigations of Bergmann[12] and Wilson[6] have already served to establish that the quaternary nitrogen interacts with the anionic site of AChE by way of Coulombic forces. The recent attempts of Thomas[13] to implicate positively charged carbon atoms in such interactions will require careful examination (see below). The operation of hydrogen-bonding effects on complex formation has been studied by Wilson[14] and also by Friess, *et al.*,[15] and it would appear that such interactions are involved at the esteratic site level. However, no quantitative data are available for ACh itself in this regard and, by inference, the conclusion is permissible that hydrogen bonds are formed between the oxygen atoms of this natural substrate and the esteratic site. Perhaps the most convincing piece of evidence that ACh is hydrogen bonded to the enzyme comes from the observation that acetylthiocholine forms a looser complex with the enzyme[16] in agreement with the reduced tendency of sulfur to form strong hydrogen bonds.

As concerns the possible involvement of van der Waals forces in the formation of addition complexes between AChE, ACh, and inhibitors, it has been common practice in the field of enzymology and drug–receptor interactions to rationalize protein–small molecule interactions in terms of the presumed universal net contribution of van der Waals forces without seemingly realizing that these forces must be of relatively rare occurrence in view of their high distance specificity.[17] The indiscriminate use of these forces in the interpretation of structure–activity relationships among enzyme inhibitors and drug–receptor interactions has served only to obscure the basic mechanisms involved and to procure an undesirable feeling of illusory comfort. The high degree of distance specificity of these forces requires that only molecules capable of producing true lock-and-key type of fits (in the classical sense) with enzymes or other proteins are likely to allow a *net positive contribution* of van der Waals forces to binding. In the great majority of cases, variations in affinity constants are interpretable in terms of hydrophobic interactions which do not include any *net* positive contribution from van der Waals attractions. The separate contributions of

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(9) B. Belleau, *J. Pharmacol. Exptl. Therap.*, in press.
(10) A. H. Beckett, *Proc. Intern. Pharmacol. Meeting, 1st, Stockholm, 1961*, **7**, 31 (1962).
(11) B. Witkop, R. C. Durant and S. L. Friess, *Experientia*, **15**, 300 (1959).
(12) F. Bergmann, A. Shimoni, and M. Wurzel, *Biochem. J.*, **63**, 684 (1956).
(13) J. Thomas, *J. Med. Pharm. Chem.*, **3**, 309 (1961); J. Thomas and W. Marlow, *ibid.*, **6**, 107 (1963).
(14) I. B. Wilson and C. Quan, *Arch. Biochem.*, **77**, 286 (1958).
(15) S. L. Friess and H. D. Baldridge, *J. Am. Chem. Soc.*, **78**, 2482 (1956).
(16) F. Bergmann, S. Rimon, and R. Segal, *Biochem. J.*, **68**, 493 (1958).
(17) L. Salem, *Can. J. Biochem. Physiol.*, **40**, 1287 (1962).

van der Waals forces and hydrophobic interactions to binding of a small molecule can be estimated readily in many cases and on the basis of such estimates (see below) it is apparent that thus far, van der Waals forces have been shown by Wilson[6] and also by Bernhard[7] to contribute positively to binding only in the case of the first two N-methyl groups of ACh. In no other case, let it be the nonpolar part of ACh or inhibitors in general, is there any evidence that such forces are operative. Since it is possible to detect the formation of true lock-and-key fits between enzymes and small molecules only when the application of these forces can be demonstrated, methods of approach to this problem are needed.

When considering the over-all physico-chemical properties of the AChE active surface, three possibilities must be considered: the surface may be either largely nonpolar in character, of intermediate polarity, or essentially polar. In the case of a nonpolar surface, the mere presence of hydrophobic substituents in a substrate or inhibitor molecule will supply a significant driving force for adsorption onto the enzyme. This driving force will have its origin exclusively in the hydrophobic interactions created by the nonpolar substituents of the substrate or inhibitor molecules and since the accommodation of these substituents by the hydrophobic binding surface occurs through the creation of as many new bonds with the nonpolar sites as are destroyed by the intruding group, no net contribution of van der Waals forces is possible. It is now recognized that the effect of hydrophobic groups on water is one of severe disturbance which is reflected in unusually large decreases in entropy on solution of the molecule in water,[18] hence the tendency for water to transfer hydrophobic groups to a more favorable environment such as the nonpolar sites of enzymes. The driving force for such transfers will fluctuate according to the extent of nonpolarity of the surface and the degree of "ideal solution" resulting from the imbedding of the intruding group into the nonpolar sites. An upper limit for the free-energy contribution of transfer forces ($\Delta F_t$) to adsorption will be set when the intruding hydrophobic group forms an "ideal solution" with the nonpolar parts of the enzyme's active surface. In practice, however, deviations from ideal behavior will be expected, and lower *but never higher values* than the limiting one will be observed for the contribution of $\Delta F_t$ to binding. On that basis only when higher values than the limiting $\Delta F_t$ can be observed will it be justifiable to call into play a net positive contribution of van der Waals forces to adsorption.

For the specific case of AChE, one must take into account the role of hydrophobic interactions in complex formation with small molecules because there exists an impressive volume of evidence that the active binding surface is nonpolar in character and hence highly susceptible to promote affinity for substrate or inhibitor molecules through hydrophobic forces. The essentially nonpolar nature of the AChE surface is revealed by its affinity for $\beta,\beta$-dimethylbutyryl acetate, a substance which acts as a relatively good substrate for the enzyme.[19] Similarly, AChE displays a gradually increasing affinity for quaternary ammonium inhibitors as the length of the hydrocarbon chain is increased from $C_1$ to $C_7$[20] (and much beyond $C_7$ as recently shown). In this latter case, it was estimated that for each additional $CH_2$ group in the alkyl chain, affinity for the enzyme increases regularly by about 300 cal., a value which is more than accountable on the basis of the contribution of $\Delta F_t$ to adsorption (see below). These examples suffice to establish the hydrophobic nature of the total active binding surface of AChE and consequently make it necessary to take into account, as a key factor contributing to affinity, the participation of hydrophobic interactions. It may

$CH_3-C(O^-)(GH)-OCH_2CH_2N(CH_3)_3$

I

II III

now become possible to elucidate the stereochemistry of enzyme-bound ACh and to define the nature of the forces contributing to the binding of ACh and structurally related inhibitors.

Because the substrate ACh is a flexible molecule devoid of steric constraint and asymmetric centers, it was essential for the purposes of our studies to select closely related rigid structures which should allow definite conclusions regarding both the relative and absolute stereospecificity of AChE. Our choice of basic structure could finally be narrowed down to the 1,3-dioxolane series of quaternary ions (II) on the basis of the following arguments: (a) the 1,3-dioxolane ring can be formally obtained by bridging the carbonyl oxygen of $\beta$-methyl-ACh with the $\beta$-methyl group (III), thus maintaining a close structural analogy with a substance acting as a substrate for the enzyme; (b) with the appropriate absolute configuration at position 4 of II, it can be inferred that C-5 of the ring ought not to interfere with the enzyme's active surface because the equivalent methyl carbon of $\beta$-methyl-ACh does not prevent the latter from behaving as a normal substrate when the asymmetric center is of the D-configuration but does when the methyl is in the L-configuration; (c) the tetrahedral character of C-2 of the dioxolane fixes the orientation of the methyl group in such a way that the orientation of the equivalent methyl group of enzyme-bound ACh may be reproduced in an appropriate stereoisomer of II; (d) the presence of the two ethereal oxygens in II should allow for hydrogen bonding with the enzyme's active sites as readily as the two similarly placed oxygens of ACh.

The only limitation of the selection of the 1,3-dioxolane ring for these studies is the impossibility of promoting binding through a nucleophilic attack (presumably by an imidazole nitrogen) at C-2, a situation which contrasts with the susceptibility of the ACh carbonyl to such attack during addition complex formation. However, there exists no positive evidence as yet that the carbonyl group of ACh is changed to the tetrahedral configuration in the complex with

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(19) D. H. Adams, *Biochim. biophys. Acta*, **3**, 1 (1949).

(20) F. Bergmann and A. Shimoni, *ibid.*, **7**, 483 (1951).

the enzyme and, in fact, recent deuterium isotope effect studies[9] with AChE would seem to favor the view that the carbonyl of ACh remains essentially trigonal in the addition complex with the enzyme. In spite of some residual uncertainty on this point, the fact remains that regardless of the resistance of C-2 of the dioxolane ring towards nucleophilic attack, the orientation of the methyl group at that position ought to bear some relationship to the orientation of the equivalent methyl group in enzyme-bound ACh. In order that the role of the oxygen atom in position 1 of the dioxolane may be evaluated in relation to complex formation, some tetrahydrofuran analogs of the 1,3-dioxolanes were tested as inhibitors of AChE. Finally, the early reports of Fourneau, *et al.*,[21] on the high cholinomimetic activity of quaternary salts of the 1,3-dioxolane series, also provided an important incentive for the initiation of the present studies especially because of the possibility of establishing novel relationships between the enzyme and its receptor counterpart.

Using erythrocyte AChE, the affinity constants of a series of 1,3-dioxolane quaternary inhibitors were measured. The synthesis and proofs of configuration of their various isomers and analogs have been reported previously.[1a,22] The results are assemble in Table I

TABLE I

INHIBITION CONSTANTS FOR AChE OF QUATERNARY INHIBITORS OF THE DIOXOLANE AND TETRAHYDROFURAN SERIES AND RELATIVE POTENCIES AS CHOLINOMIMETICS ON THE GUINEA PIG ILEUM

$R_2$, $R_1$ (C-2) – 1,3-dioxolane – $CH_2\overset{+}{N}(CH_3)_3$

| Compd. | $R_1$ | $R_2$ | Configuration | $K_i \times 10^5$ *M* | Relative affinities | Relative potencies |
|---|---|---|---|---|---|---|
| IV | H | H | DL | 45.0 | 100 | 0.1 |
| V | $CH_3$ | H | DL-*cis* | 7.5 | 600 | 100 |
| VI | $CH_3$ | H | L-(+)-*cis* | 4.1 | 1100 | 625 |
| VII | $CH_3$ | H | D-(−)-*cis* | 67.0 | 67 | 6 |
| VIII | H | $CH_3$ | DL-*trans* | 23.0 | 200 | 20 |
| IX | $CH_3$ | $CH_3$ | DL | 60.0 | 75 | 0.5 |
| X | $CH_3$ | $CH_3$ | D-(−) | 85.0 | 53 | 0.1 |
| XI | $CCl_3$ | H | DL-*cis* | 1.1 | 4100 | 1 |
| XII | H | $CCl_3$ | DL-*trans* | 7.4 | 610 | 0.1 |
| XIII | tetrahydrofuran–$CH_2\overset{+}{N}Me_3$ | | DL | 49.0 | 91 | ... |
| XIV | $CH_3$–tetrahydrofuran–$CH_2\overset{+}{N}Me_3$ | | DL-*cis* | 17.0 | 265 | |
| Formylcholine | | | ... | $K_m$ = 350 | 12 | 5 |
| ACh | | | ... | $K_m$ = 45 | 100 | 100 |

where relative cholinomimetic activities reported earlier are included for easy reference. It was established that all of these compounds behave as competitive inhibitors of the enzyme.

## Results and Discussion

**A. Stereospecificity of AChE and Muscarinic Cholinergic Receptors.**—Table I shows that AChE displays both relative and absolute stereospecificity towards the 1,3-dioxolane series of inhibitors. This stereospecificity attains its highest degree towards one optical form of *cis*-2-methyl-4-trimethylammoniummethyl-1,3-dioxolane iodide (VI), the L-(+) isomer of which represents the optimum configuration for maximum inhibitory power among the analog series most closely related to ACh itself. This isomer (VI) possesses an affinity for AChE which is approximately 10 times greater than that of ACh itself; however, the best inhibitor of the series is the *cis*-trichloromethyl derivative (XI), a compound which is rather distantly related to ACh. Thus, AChE displays a high degree of absolute stereospecificity, but possesses moderate relative stereospecificity and little structural specificity towards the dioxolane series of inhibitors. A most striking feature is the influence of the C-2 methyl substituent on affinity for the enzyme, a phenomenon which is discussed below. Optimal cholinomimetic activity is associated with the *cis*-L-(+) derivative (VI), but is relatively independent of affinity for AChE since the trichloromethyl compound (XI) is weakly active as a cholinomimetic. Similarly to AChE, however, the receptor is found to display a high degree of absolute stereospecificity but limited structural specificity towards this series of stimulants. As would have been expected, the absolute configuration about C-4 of the best inhibitors or stimulants is the same as the configuration of the β-carbon of D-β-methyl-ACh, the stereoisomer acting as a substrate for the enzyme and also as the active cholinomimetic on muscarinic receptors. C-5 of inhibitor VI may therefore not participate nor interfere to any appreciable degree in the binding phenomenon. However, the C-2 methyl substituent, when in the configuration fixed by inhibitor VI, has a dramatic influence on both the affinity of the dioxolane ring for AChE and muscarinic potency. Indeed the cholinergic receptor and AChE display identical patterns of stereospecificity towards this class of quaternary ions and a similar predilection for a properly oriented methyl group which is structurally equivalent to that of the ester methyl of ACh. These results suggest that the effects of the C-2 methyl group of VI on affinity may be attributed to a faithful reproduction of the natural steric orientation of the equivalent methyl group of enzyme- or receptor-bound ACh. That this is probably the case is shown below. Finally, the influence of the C-2 methyl group of inhibitor VI on affinity and potency is conditioned to a high degree by the presence of the two oxygen atoms of the dioxolane ring, since the tetrahydrofuran analog XIV (deoxy-DL-muscarine) is about one third as active as the dioxolane V. Whereas the *cis*-methyl group increases affinity by a factor of 3 in the tetrahydrofuran series, it increases it by a factor of 6 in the dioxolane series. A perfect fit of the methyl group on AChE is thus conditioned by interactions with two oxygen atoms as in the case for ACh itself.

On the basis of these results, the conclusion emerges that both AChE and the muscarinic receptor display similar absolute and relative stereospecificities and that the configuration of inhibitor VI must bear some important relationship to the conformation of enzyme- and receptor-bound ACh.

**Interpretation of Structure–Activity Relationships.**—Table I shows that the effect of the C-2 substituents of the dioxolane series on affinity is erratic. If the *cis* configuration is optimal for binding, then a lower affinity of the *trans* isomer (VIII) than for the parent desmethyl

(21) E. Fourneau, D. Bovet, F. Bovet, and G. Montezin, *Bull. soc. chim. biol.*, **26**, 516 (1944).

(22) D. J. Triggle and B. Belleau, *Can. J. Chem.*, **40**, 1201 (1962).

analog (IV) would be expected since the methyl group should produce nuisance to binding when in the *trans* arrangement. However, this is not the case, the *trans* isomer VIII possesses a higher affinity than the desmethyl analog. It can therefore be expected that the dimethyl analog (IX) which includes both a *cis*- and a *trans*-methyl group should now have a higher affinity than either the *cis*- or *trans*-monomethyl compounds V and VIII. In fact, the dimethyl inhibitor (IX) possesses an affinity which is even *lower* than that of the parent desmethyl analog (IV). Also, if the *cis* inhibitor (VI) interacts optimally with the enzyme, why then should the *cis*-trichloromethyl analog (XI) have a higher affinity for the enzyme? These apparently conflicting results can be interpreted through a consideration of the nature of the forces involved in binding onto the enzyme, if one takes into account the fact that the adsorption of nonpolar groups onto the enzyme is conditioned primarily by the operation of hydrophobic forces and to a restricted extent by van der Waals forces. The early classical investigations of Cohn and Edsall[23] provide a quantitative basis for the evaluation of the separate contribution of hydrophobic interactions to the transfer of methylene and methyl groups of amino acids from an aqueous phase to a nonpolar one. Through quantitative solubility measurements, these authors have established that the transfer of a methyl group from water to a nonaqueous phase is accompanied by a free-energy change of −730 cal. Since the AChE surface is highly nonpolar in character (see above), it is clear that the hydrophobic forces exerted on the nonpolar C-2 methyl groups of the dioxolane inhibitors can contribute largely to affinity for the enzyme, the upper limit of 730 cal./methyl group being sufficient to shift binding equilibrium by a factor of as much as 3. In practice, however, it will be expected that this upper limit may be attained only when "solution" of the methyl group by the enzyme surface follows ideal behavior. In other words, maximum contribution of $\Delta F_t$ to binding will occur when the intruding alkyl group breaks as many bonds in the nonpolar network of AChE as it creates new ones with the enzyme. Of great significance in this connection was the discovery by Cohn and Edsall[23] that the total free energy of transfer of a nonpolar side chain is made of the sum of each separate contribution of the component parts and is independent of the presence of polar parts in the molecule. Hence, the values computed by these authors are applicable at least in principle to the above series of quaternary ion inhibitors and this as long as the AChE total binding surface is essentially nonpolar in character. Because no data were available on the quantitative role of hydrophobic forces in the case of the trichloromethyl substituent, the appropriate measurements using Cohn and Edsall's method were made using the readily available inhibitors XI and XII. The free energy of binding of the key inhibitors was computed using the classical relationship $\Delta F_i = -RT \ln K_i$; theoretical estimates of $\Delta F_i$ for each inhibitor can be made by adding to the basic $\Delta F_i$ value for the unsubstituted inhibitor IV the expected contribution of $\Delta F_t$ to binding produced by the introduction of a nonpolar substituent. For the case of the *gem*-dimethyl substituted inhibitor (IX), the value of $\Delta F_t$ was also estimated according to Cohn and Edsall. The results are assembled in Table II; for all the compounds tested, excepting inhibitor VI, the variations in affinity constants are all interpretable in terms of variations in the contribution of $\Delta F_t$ to binding; however, transfer of hydrophobic groups to the nonpolar enzyme surface does not necessarily occur under "ideal" conditions, in the sense that "solution" of the substituent can often deviate from ideal behavior (see above), hence, the positive discrepancies in the theoretically estimated values of $\Delta F_i$. A negative discrepancy such as is obtained with inhibitor VI is unaccountable on the basis of the contribution of $\Delta F_t$ alone, so that the net participation of the highly distance-specific van der Waals forces must now be called into play in order to account for the affinity of VI for the enzyme. On the basis of Salem's theoretical treatment,[17] a maximum value of 600 cal./$CH_3$ group is obtained for the free-energy contribution of van der Waals interactions with suitably oriented methylene groups on the enzyme. This estimate is in excellent agreement with the observed value of 535 cal. (Table II) for the 2-methyl group of VI. The still higher affinity of the trichloromethyl compound does not reflect, however, the operation of van der Waals forces, the application of which remains an exclusive privilege of the L-(+)-*cis* inhibitor (VI). On that basis, one can distinguish two entirely different modes of inhibitor interactions with AChE: (a) those which are conditioned by the operation of hydrophobic forces alone, thereby necessitating a *nonspecific accommodative perturbation of the network of nonpolar chains* corresponding to a more or less ideal "dissolution" of a nonpolar substituent of an inhibitor or substrate molecule; (b) those which allow the application of the distance-specific van der Waals attractions in addition to hydrophobic forces, thus conducive to a *highly specific perturbation of the nonpolar surface* of the enzyme. In this case, new bonds are formed with the enzyme and this in the absence of any or little disruption of pre-existing bonds in the nonpolar network. Only in such a situation can one describe the closeness of fit between enzyme and a small molecule as belonging to the lock-and-key type of fit, because of the extreme distance specificity of van der

TABLE II

FREE ENERGY OF BINDING OF DIOXOLANE INHIBITORS[a]

| Compd. | $\Delta F_i$ obsd. | $\Delta F_i$ calcd. | $\Delta F_i$, calcd. − obsd. | $\Delta F_t$ for 2-substituent | $\Delta F_w$ |
|---|---|---|---|---|---|
| IV | 4568 | | | | |
| VI | 5923 | 5298 | −535 | 730[b] | 600 |
| VIII | 4996 | 5298 | +302 | 730[b] | 0 |
| IX | 4520 | 4918 | +398 | 350[c] | 0 |
| XI | 6761 | 6848 | +87 | 2280[c] | 0 |
| XII | 5630 | 6908 | +1278 | 2340[c] | 0 |
| XIII | 4500 | | | | |
| XIV | 5125 | 5230 | +105 | 730[b] | 0 |

[a] $\Delta F_i$ = total free energy of binding in cal./mole, $\Delta F_t$ = free energy of transfer of 2-substituents from an aqueous phase to a nonpolar one, $\Delta F_w$ = free energy of binding contributed by van der Waals attractions, $\Delta F_i$(calcd.) = 4568 ($\Delta F_i$ for IV) + $\Delta F_t$, or 4500 ($\Delta F_i$ for XIII) + $\Delta F_t$. [b] Value given by Cohn and Edsall.[23] [c] Determined by Cohn and Edsall's method.[23]

(23) E. J. Cohn and J. T. Edsall, "Proteins, Aminoacids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, Chapter 9.

Waals forces.[17] It is significant that only inhibitor VI should allow the application of these forces to the C-2 methyl group. This observation confirms the conclusion that the steric orientation of that group must correspond closely to a natural and special predilection of the enzyme surface for the geometry of inhibitor VI. Since a lock-and-key type of fit applies to the C-2 methyl of VI and that such fits are generally believed to be a privilege of natural substrates, the conclusion is also confirmed that inhibitor VI may reproduce closely the preferred conformation assumed by ACh when bound onto the enzyme. If this were the case, it would be expected that the ester methyl group of ACh must also allow the application of distance-specific van der Waals forces in the Michaelis complex with the enzyme. This could be verified through a comparison of the affinity constants of formylcholine and ACh. After appropriate corrections for the spontaneous hydrolysis of formylcholine, a value of 1.2 kcal. for the free-energy contribution to binding of the acetyl methyl group was obtained. The methyl groups of both ACh and inhibitor VI therefore lead to the formation of lock-and-key fits with the esteratic portion of the enzyme. We conclude on that basis that the conformation of enzyme-bound ACh most probably corresponds to the geometry of the unique inhibitor VI.

It now becomes possible to rationalize structure–activity relationships at the level of the muscarinic cholinergic receptor. In agreement with the finding that VI interacts with AChE in an ACh-like manner, it would be expected that the same should apply to other natural bioreceptors that are specific for ACh. The fact that VI largely surpasses ACh in potency at the cholinergic receptor level substantiates these expectations. However, all those inhibitors whose affinities are conditioned by nonspecific accommodative perturbations do not interact in an ACh-like manner with the receptor and therefore cannot act as efficient inducers of the specific receptor perturbation that is required for the initiation of a physiological stimulus. Moreover, those molecules whose affinities for the protein surface do not benefit from the operation of van der Waals attractions or hydrophobic forces should be less effective as stimulants. This is the case, for instance, for the unsubstituted dioxolane IV and its 2,2-dimethyl analog (IX). The low affinity and low potency of the latter is ascribable to the unfavorable $\Delta F_t$ for a *gem*-dimethyl group (Table III), a phenomenon related to the fact that steric hindrance prevents the nonpolar chains of the protein from interacting optimally with all of the CH bonds of the methyl groups. As a consequence, fewer new bonds are created between the methyl groups and the enzyme or receptor than are destroyed by them. Hence the unfavorable energy of binding for the *gem*-dimethyl drug. This interpretation is in line with the known detrimental effects of methyl branching on the van der Waals interactions between fatty acid chains.[17] The low muscarinic potency but high affinity for AChE of the trichloromethyl drugs (XI and XII) is a reflection of the operation of $\Delta F_t$ which is conditioned by nonspecific accommodative perturbations of the nonpolar chains of the protein. Such perturbations bear little relationship to the specific conformational changes induced by ACh or the inhibitor VI and account for the inactivity as stimulants of the trichloromethyl compounds.

TABLE III[a]

| Compd. | $N_W \times 10^{-8}$ | $N_A \times 10^{-3}$ | log $N_A/N_W$ | $\Delta F_t$, cal. |
|---|---|---|---|---|
| IV | 30.8 | 4.9 | −0.70 | |
| IX | 7.35 | 2.7 | −0.44 | 350 |
| XI | 0.13 | 1.75 | 0.93 | 2280 |
| XII | 0.63 | 6.65 | 1.02 | 2340 |

[a] $N_W$ = solubility in water at 23° (mole fraction), $N_A$ = solubility in ethanol at 23° (mole fraction), $\Delta F_t$ = free energy of transfer for the 2-substituent(s) = free energy of transfer of substituted compound − free energy of transfer for compound IV.

To summarize, it can now be recognized that the binding of quaternary ions onto AChE or its receptor counterpart can have quite divergent effects on the conformation of the protein; either little disruption of pre-existing bonds between the nonpolar chains occurs or more or less profound perturbations of the hydrophobic network take place when nonpolar substituents are brought into contact with the enzyme's active surface. It is probable that this is what forms the basis of structure–activity relationships at the receptor level, stimulant activity being a characteristic of those ions which do not induce undesirable accommodative perturbations of the protein's binding surface. These considerations form the basis of a molecular theory of drug action which is proposed in an accompanying paper.[24]

In a comparison of our results in the dioxolane series of AChE inhibitors with those of Friess, *et al.*,[11] in the muscarine series of isomers, the affinity of DL-deoxymuscarine (XIV) for AChE is higher than that of DL-muscarine itself. This suggests that the hydroxyl function of the latter hinders rather than promotes complex formation. Of interest was the observation that similarly to the dioxolane series, a *cis* arrangement of the substituents on the ring of DL-muscarine is optimal for high affinity for the enzyme, although this requirement is not as critical as in the dioxolane series. Curiously enough, in the DL-muscarine series, the *trans* configuration appears to be more favorable to binding than the *cis* arrangement. As noted above, the effect of the *cis*-methyl group on affinity of deoxymuscarine for AChE does not suggest any positive contribution from van der Waals forces to binding since hydrophobic forces alone account for the effect of the methyl group on affinity (about 700 cal./$CH_3$). It is therefore likely that in both the muscarine and muscarone series, affinity for AChE may be largely controlled by hydrophobic forces, a possibility which makes comparisons with the dioxolane isomers rather meaningless since in that series, the L-(+)-*cis* isomer (VI) allows the application of van der Waals attractions to the 2-methyl substituent. The probable participation of $\Delta F_t$ alone in the muscarine series can only serve to mask the inherent stereospecificity of the enzyme. Friess, *et al.*,[11] also reported that acetylation of the hydroxyl function of the muscarine isomers uniformly increases affinity for the enzyme by a factor of 2–3. The magnitude of the effect (the binding energies increasing by about 500 to 700 cal.) strongly suggests that hydrophobic forces are chiefly responsible for the increased affinities of the acetylmuscarines and that no

(24) B. Belleau, *J. Med. Chem.*, 7, 776 (1964).

specific bonds, other than "dissolution" into the nonpolar network, are formed between the acetyl groups and the enzyme's active sites. The fact that deoxymuscarine (XIV) possesses a comparable affinity strongly supports this view. The partial inversion of the optical specificity of receptors towards the muscarone enantiomers has been interpreted in another paper.[1a] However, the receptor and AChE display similar absolute and relative stereospecificities towards both the muscarine and dioxolane series of isomers. The bearing of these considerations on the factors intervening in complex formation with AChE is taken advantage of in the elaboration of the macromolecular perturbation theory of drug action.[24]

**Role of the Quaternary Nitrogen of Drugs in Complex Formation.**—The investigations of Wilson[6] on the role of the N-methyl groups of ACh on affinity for AChE have revealed that each of the first two methyl groups increases affinity for the enzyme by a factor of 7, whereas the third methyl group is without effect on binding, although its presence causes a marked increase in the entropy of the complex. This factor of 7 on the affinity corresponds to a free-energy contribution to binding of 1.3 kcal./methyl group, a value which was tentatively rationalized by Bernhard[7] and also Wilson[6] on the basis of the operation of van der Waals attractions. However, using Salem's calculations as a basis,[17] the contributions of van der Waals forces to the binding of a single methyl group cannot exceed 600 cal., leaving an excess of 700 cal. unaccounted for. If one takes into account the fact that hydrophobic forces alone should contribute about 730 cal. of driving force for binding, one arrives at a maximum total energy of interaction of 1.33 kcal./methyl group, as long as the AChE surface is largely hydrophobic in character. Evidence for this view has been discussed above. The forces contributing to the binding of the N-methyl groups and the ester methyl group of ACh or the 2-methyl group of inhibitor VI are of an identical magnitude. The operation of hydrophobic forces in the adsorption of the N-methyl groups clearly establishes that the environment of the anionic site of AChE is also highly hydrophobic. This allows the prediction that groups bulkier than N-methyl would also induce nonspecific accommodative perturbations of the network of nonpolar chains at the periphery of the anionic binding site. It follows that bulky substituents on the nitrogen of quaternary salts will cause deformation of AChE or the receptor protein counterpart. In this latter case, the effect would be reflected in a reduction of stimulating activity upon complex formation.

The absence of an effect on binding in the case of the third methyl group and the dramatic effect on the entropy of the complex produced by the same group are most interesting. Clearly, this methyl group has a profound effect on the structure of the protein and a large increase in the flexibility of some parts of the protein must occur as a result of its presence. In our opinion, this effect can best be rationalized as follows. When in the aqueous solvent, the N-methyl group exerts hydrophobic interactions and thus repels water molecules. The free energy of this interaction amounts to about 730 cal. Since the same methyl group does not contribute positively to affinity when on the enzyme surface, it must still be in an aqueous or highly polar environment, where as many water molecules are repelled as in the solvent water. The effect of the methyl group on the enzyme may be to expel water molecules from the active sites, *thus creating an anhydrous environment for nucleophilic attack of the ester carbonyl.* It is known that the nucleophilicity of various nucleophiles is increased in the absence of solvation.[25] This serves to explain the marked increase in the rate of acetylation of the enzyme produced by the third N-methyl group of ACh when comparison is made with N-dimethylaminoethyl acetate. It would seem therefore that one of the basic reasons why hydrolytic enzymes are so successful as catalysts may lie in their ability to provide an anhydrous medium (through appropriate conformational changes) for efficient nucleophilic attack of the reactive centers of substrates. Concrete evidence for this generalization is being sought actively.

The investigations of Bergmann[12] and Wilson[2,6] taken in conjunction with the considerations set forth in this paper rationalize adequately the mechanism of interaction of quaternary ions with the anionic site of AChE. However, an alternative mechanism has been recently suggested by Thomas, *et al.*,[13] who attempted to show that the Coulombic attraction between quaternary ions and a counter ion on the enzyme surface would no longer involve the focus of the charge on the nitrogen, but rather the carbon atoms acting as substituents. It was postulated that these carbon atoms carry positive charges, an effect thought to arise from an electron delocalization produced by the strong electronegative pull of the positive nitrogen. Evidence for this mode of interaction between quaternary ions and AChE is thought to have been produced by taking advantage of special steric effects and other presumed electronic effects. The mechanism of binding of quaternary ions as proposed by Thomas should be considered with circumspection for the following reasons. (a) The theoretical treatment of Pauling[26] which is cited in support of the hypothesis that the carbon atoms attached to a quaternary nitrogen share the positive charge of the nitrogen was actually applied by Pauling to the ammonium ion and not to quaternary ions. (b) Should any residual positive charge be present on the substituent carbon atoms, some degree of hydration of the quaternary ion would be a normal and expectable consequence of contact with water molecules. In fact, all presently available data clearly support the view that such ions are not hydrated in water.[27] Such ions are generally considered essentially hydrophobic in nature.[18] (c) The case of the anilinium ion which was assumed by Thomas to provide for enhanced delocalization of the positive charge through electronic interaction with the phenyl ring is unacceptable on the basis of the well-known fact that positively charged nitrogen on a benzene ring does not alter the ultraviolet absorption characteristics of the aromatic chromophore.[28] The

(25) W. M. Weaver and J. D. Hutchison, *J. Am. Chem. Soc.*, **86**, 261 (1964).

(26) L. Pauling, "The Nature of the Chemical Bond," Cornell University Press, Ithaca, N. Y., 1948, p. 71.

(27) J. Desnoyers, Ph.D. Thesis, University of Ottawa, 1961.

(28) F. A. Matsen, "Technique of Organic Chemistry," Vol. IX, A. Weissberger and W. West, Ed., Interscience Publishers, Inc., New York, N. Y., 1956, p. 671.

gradual decrease in affinity constants for AChE produced by separation of the positive nitrogen from the phenyl ring through the insertion of methylene groups is probably the simple reflection of a gradual decrease in the ability of the benzene ring to form $\pi$-bonds with the enzyme's active sites. It is likely that the distance between the positive nitrogen and the $\pi$-electron cloud is critical for optimal interaction with the enzyme. (d) Should the substituent carbon atoms of quaternary nitrogen share the positive charge, the N–C bonds would have increased polarizability and hence the bond length should be different from that in the corresponding tertiary amines. However, this is not the case; both types of amine derivatives have nearly identical N–C bond lengths.[29] It is most likely that the results of Thomas are relevant to the operation of hydrophobic forces and $\pi$-interactions with the enzyme's active sites.

## Experimental[30]

**Materials.**—Except for compounds XI and XII, the 1,3-dioxolane inhibitors (Table I) have been described previously.[1a,22] Compounds XIII and XIV were prepared according to the literature.[31]

**DL-*cis*-2-Trichloromethyl-4-trimethylammoniummethyl-1,3-dioxolane Iodide (XI).**—Five grams of DL-*cis*-2-trichloromethyl-4-tosyloxymethyl-1,3-dioxolane[22] was treated with excess anhydrous dimethylamine in 50 ml. of dimethyl sulfoxide. After standing overnight, followed by heating for 1 hr. at 100°, the solvent was evaporated *in vacuo*, the residue was mixed with saturated aqueous potassium carbonate, and the amine was extracted with ether. The ether was dried and evaporated, and the residue was distilled *in vacuo* to give **DL-*cis*-2-trichloromethyl-4-dimethylaminomethyl-1,3-dioxolane**, b.p. 115° (10 mm.) (85% yield).

*Anal.* Calcd. for $C_7H_{12}Cl_3NO_2$: C, 33.80; H, 4.82. Found: C, 33.64; H, 4.96.

The **methiodide** XI was obtained in the usual manner and was recrystallized for ethanol, m.p. 243–245°.

*Anal.* Calcd. for $C_8H_{15}Cl_3INO_2$: C, 24.58; H, 3.84. Found: C, 24.41; H, 3.70.

**DL-*trans*-2-Trichloromethyl-4-trimethylammoniummethyl-1,3-dioxolane Iodide (XII).**—The previously described DL-*trans*-2-trichloromethyl-4-tosyloxymethyl-1,3-dioxolane[22] was carried through the same procedures described above in the case of the preparation of XI. In this manner, the DL-*trans*-2-trichloromethyl-4-dimethylaminomethyl-1,3-dioxolane, b.p. 110–115° (12 mm.), was obtained in 75% yield.

*Anal.* Calcd. for $C_7H_{12}Cl_3NO_2$: C, 33.80; H, 4.82. Found: C, 33.95; H, 4.66.

The **methiodide** XII was recrystallized from ethanol, m.p. 195°.

*Anal.* Calcd. for $C_8H_{15}Cl_3INO_2$: C, 24.58; H, 3.84. Found: C, 24.68; H, 3.78.

**Formylcholine Bromide.**—A mixture of acetic anhydride (10 ml.) in excess formic acid (98%) was prepared and 12 g. of 2-bromoethanol was added. After several hours at room temperature, the 2-bromoethyl formate was isolated in the usual manner. The ester thus obtained was treated with excess anhydrous trimethylamine in benzene at 100° in a pressure bottle. After 10 hr., the solid was collected and recrystallized from absolute ethanol, m.p. 147–149°.

*Anal.* Calcd. for $C_6H_{14}BrNO_2$: Br, 37.73; sapon. equiv., 212. Found: Br, 37.85; sapon. equiv., 205.

**Methods.**—Bovine erythrocyte acetylcholinesterase from a commercial source (Nutritional Biochemicals Corp.) was used. The rates of acetylcholine bromide hydrolysis were measured with an automatic titrator using a pH stat equipped with a recorder (Copenhagen Radiometer, Ole Dich recorder and syringe attachment). The reaction medium consisted of glass-distilled water, 0.15 $M$ in NaCl and 0.015 $M$ in $MgCl_2$; the temperature was kept at 25 ± 0.1° and the pH at 7.4. A $CO_2$-free nitrogen atmosphere was maintained throughout. The value of $K_m$ for ACh was consistently $4.5 \pm 0.1 \times 10^{-4}$ $M$ in agreement with published data. The determination of inhibition constants was accomplished in the usual manner using four different inhibitor concentrations and $4 \times 10^{-3}$ $M$ ACh. The $K_i$ values were calculated using the relationship $K_i = 0.09 \times C_{50}$ where $C_{50}$ represents the concentration of inhibitor required to produce 50% inhibition of the enzyme; this relationship is derived from the following equation.[32]

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

Using an inhibitor concentration to produce 25% inhibition, initial velocities for four ACh concentrations inferior to the $K_m$ were determined and conventional Lineweaver–Burk plots[33] were constructed. All curves thus obtained had a common extrapolated intercept, thus establishing that all the inhibitors of Table I act competitively. Three separate determinations of $K_i$ were made.

**Solubility Determinations.**—The procedure advocated by Cohn and Edsall[23] was applied. Excess solute IX, XI, and XII was added to distilled water and to absolute ethanol. The mixtures were allowed to equilibrate at room temperature (23°) by shaking for 20 hr. and then centrifuged. An aliquot of the clear supernatants was drawn and the respective densities of the solutions measured. The concentrations thus obtained are given in Table III. The calculated values for the free energy of transfers can only be approximate since the assumption is implicit that the activity coefficients are close to unity. However, due to the low solubilities of IX, XI, and XII, the approximation should be a good one. On the other hand, the higher solubility of IV probably introduces larger deviations from ideal behavior.

(29) Tables of Interatomic Distances and Configuration in Molecules and Ions, Special Publication No. 11, The Chemical Society (London), Burlington House, London, 1958, pp. M156, M175, M115.

(30) Microanalyses by Midwest Microlab, Indianapolis, Ind. Melting points were determined on a Kofler hot stage and are corrected.

(31) A. C. Cope and E. E. Schweizer, *J. Am. Chem. Soc.*, **81**, 4577 (1959).

(32) J. A. Cohen and R. A. Oosterbaan, "Handbuch der Experimentellen Pharmakologie," Vol. XV, G. B. Koelle, Sub-Ed., Springer-Verlag, Berlin, 1963, Chapter 7.

(33) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).