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Molecular Mechanisms for Hydrolytic Enzyme Action. II. Inhibition of Acetylcholinesterase by Excess Substrate

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Rate equations are developed for inhibition by substrate, in the presence of a competitive inhibitor, for cases where the substrate and inhibitor may add on to the acetyl enzyme or the Michaelis complex. It is shown how the equations may be used to study the mechanism of substrate inhibition. An experimental investigation of the effect of *cis*-2-dimethylaminocyclohexanol on the inhibition of acetylcholinesterase by acetylcholine, together with similar studies in the literature with choline and prostigmine, showed that the substrate inhibits by combining with the acetyl enzyme. The molecular mechanism of substrate inhibition is discussed.

Introduction

High concentrations of acetylcholine are known to inhibit its hydrolysis by acetylcholinesterase. In general the phenomenon of inhibition by excess substrate has been attributed to the formation of an inactive ternary complex of the enzyme with two molecules of the substrate. (With two-substrate reactions involving a compulsory order of addition of the substrates to the enzyme surface, an alternative mechanism is possible.[1]) According to a mechanism (i) proposed for acetylcholinesterase,[2] the enzyme possesses, near the catalytic esteratic site, two anionic groups that can interact with the positive charge of the quaternary nitrogen atom in acetylcholine. Two acetylcholine molecules may be bound at these sites in such a way that neither substrate molecule is able to interact in the required manner with the esteratic site, and cleavage of the ester bond in acetylcholine hence does not occur. In another suggested mechanism (ii) only one anionic site is involved; one molecule of substrate becomes attached to the anionic site and neither molecule is then able to react further.[3] Still another possibility (iii) is that a molecule of substrate becomes attached to the acetylated enzyme and blocks deacetylation.[4]

It was shown in Part I of this series[5] that a substance containing a quaternary nitrogen atom may inhibit not only by adding on to the free enzyme at the same site as the substrate but also by adding on to the acyl-enzyme, in which the anionic site is free. If the inhibitor possesses an appropriately placed electronegative grouping,[6] this interacts with a catalytic grouping in the esteratic site and blocks deacylation. Such substances do not inhibit through attachment to the Michaelis complex. In view of this, substrate inhibition may similarly be due to the addition of a molecule of acetylcholine to the acetyl-enzyme as in mechanism (iii). Possibly the carbonyl oxygen interacts with the esteratic site, blocking deacetylation.

The reasoning which has suggested that acetylcholine may inhibit through attachment to the acetyl enzyme also suggests that other compounds containing a quaternary nitrogen atom, such as choline, should also become attached to the acetyl enzyme. The latter compound, however, lacks a locus of high electron density. For this reason the suggestion is raised that such a compound might fail to inhibit deacetylation even though it is attached to the intermediate in question.

This appears to be particularly likely since the acylation process, which involves the splitting of the bond joining the acetate and choline portions of the substrate, would give rise to an acetyl-enzyme containing an attached choline molecule. It does so because the forces between the positive nitrogen of choline and the anionic site continue to operate in the acetyl-enzyme. This arrangement would result in a very inefficient catalytic mechanism if choline was inhibitory.

Friess and Baldridge[7] showed that the optimal separation of the ammonium ion and the electronegative locus of the inhibitor is 2.5 Å. or less. Consequently certain inhibitors of acetylcholinesterase, such as prostigmine and eserine, may be too large to make suitable contact with the esteratic site. This might be due to the presence of the acetyl grouping in the acyl-enzyme. In addition, of course, such inhibitors might lack a suitably placed electronegative center. These considerations would lead one to expect that among the inhibitors of acetylcholinesterase which contain a quaternary nitrogen atom all could be bound to the free and acetyl enzymes; certain of these inhibitors would block deacetylation, while others, because of size or the position of an electronegative grouping, would not do so.

Theory.—The suggestion (iii) that substrate inhibition occurs through combination of the substrate with the acetyl enzyme, together with the suggestion that certain inhibitors may become attached to this enzyme species without blocking deacylation, leads to the formulation of a scheme for the inhibition of acetylcholinesterase by compounds containing a positive nitrogen atom. Such a formulation is shown in Fig. 1. The scheme is made quite general by allowing the complex between the acetyl enzyme (ES′) and the substrate (S) to break down to form products with a rate defined as bk_3, where b is a factor equal to zero or larger. Neither substrate (S) nor inhibitor (I) is able to add on to the Michaelis complex (ES), but both can add on to the acetyl-enzyme (ES′). The inhibitor can also add on to the free enzyme (E).

(1) R. M. Krupka and K. J. Laidler, *Can. J. Biochem. Physiol.*, **38**, 1185 (1960).

(2) F. Bergmann, I. B. Wilson and D. Nachmansohn, *Biochim. Biophys. Acta*, **6**, 217 (1950).

(3) E. A. Zeller and A. Bissegger, *Helv. Chim. Acta*, **26**, 1619 (1943).

(4) I. B. Wilson and E. Cabib, THIS JOURNAL, **78**, 202 (1956).

(5) R. M. Krupka and K. J. Laidler, *ibid.*, **83**, 000 (1961).

(6) S. L. Friess and W. J. McCarville, *ibid.*, **76**, 1363 (1954).

(7) S. L. Friess and H. D. Baldridge, *ibid.*, **78**, 199 (1956).

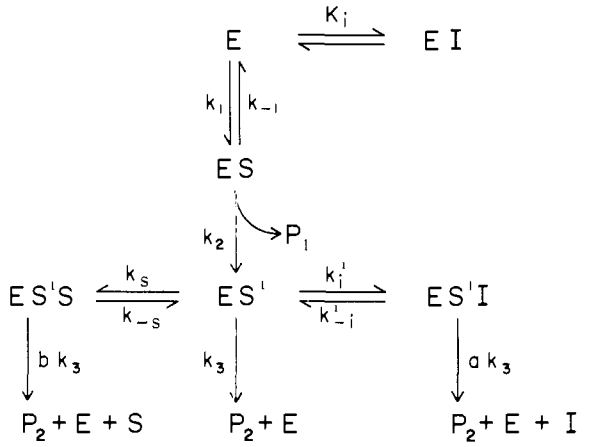

Fig. 1.—Reaction scheme for substrate inhibition: in the presence of an inhibitor I which can combine with the free enzyme and the acyl enzyme but not with the addition complex. This scheme appears to apply to the data.

Rate equations for substrate inhibition will now be derived on the basis of this formulation; it should then be possible to determine whether or not the scheme is in agreement with experimental findings. Application of the steady-state treatment to the scheme in Fig. 1 gives rise to

$$v = \frac{k_2[E]_0\bar{K}[S]}{1 + K_i[I] + \bar{K}[S]\left\{1 + \frac{k_2(1 + \bar{K}_s[S] + \beta\bar{K}_i[I])}{k_3(1 + b\bar{K}_s[S] + a\beta\bar{K}_i[I])}\right\}} \quad (1)$$

where

$$\beta\bar{K}_i = k'_i/(k'_{-i} + ak_3), \qquad \bar{K} = \frac{k_1}{k - 1 + k_2} \text{ and}$$

$$\bar{K}_s = \frac{k_s}{k - s + bk_3}$$

Assuming that b is sufficiently small so that the term $b\bar{K}_s[S]$ can be neglected, the expression for the optimum substrate concentration can be determined by setting $dv/d[S]$ equal to zero. It is then found that the optimum substrate concentration in the presence of inhibitor is given by

$$[S]^I_{opt} = \left\{\frac{k_3(1 + K_i[I])(1 + a\beta\bar{K}_i[I])}{k_2\bar{K}K_s}\right\}^{1/2} \quad (2)$$

where K_s is approximately equal to k_s/k_{-s}. If a and β are unity and $\bar{K}_i = K_i$, equation 2 becomes

$$[S]_{opt} = \left\{\frac{k_3}{k_2\bar{K}K_s}\right\}^{1/2}(1 + K_i[I]) \quad (3)$$

In the absence of inhibitor ($[I] = 0$) equation 2 becomes

$$[S]_{opt} = \left\{\frac{k_3}{k_2\bar{K}K_s}\right\}^{1/2} \quad (4)$$

Under these conditions the ratio of the substrate optimum in the presence of the inhibitor to that in its absence is

$$\frac{[S]^I_{opt}}{[S]_{opt}} = 1 + K_i[I] \quad (5)$$

Similar reasoning shows that if a is zero

$$\frac{[S]^I_{opt}}{[S]_{opt}} = (1 + K_i[I])^{1/2} \quad (6)$$

It will now be useful to compare this scheme (Fig. 1) with that shown in Fig. 2. The latter represents cases i and ii described in the Introduction. For simplicity the acetyl enzyme is not shown. In this scheme the substrate becomes attached to the Michaelis complex but not to the acetyl-enzyme. Application of the steady-state treatment shows that in the presence of the inhibitor the reaction velocity is

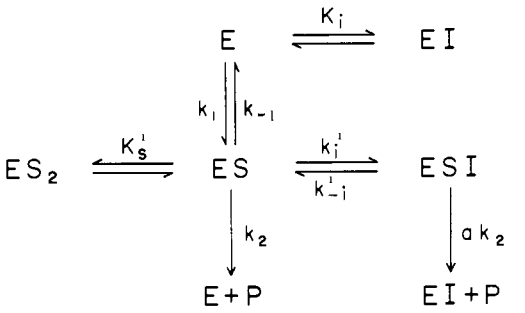

Fig. 2.—Another scheme for substrate inhibition, in which the inhibitor combines with the addition complex; the data are inconsistent with this scheme.

$$v = \frac{k_2[E]_0[S]\left\{\frac{k_1}{k_{-1} + k_2 + ak_2\alpha\bar{K}_i[I]}\right\}}{1 + K_i[I] + \frac{k_1(1 + K_s'[S] + \alpha\bar{K}_i[I])[S]}{(k_{-1} + k_2 + ak_2\alpha\bar{K}_i(I))}} \quad (7)$$

where $\alpha\bar{K}_i = k_i'/(k'_{-i} + ak_3)$. The optimum substrate concentration can now be expressed in terms of the inhibitor concentration for three different cases. In the first case the complex ESI is not formed; that is, α is equal to zero. Here

$$[S]_{opt} = \left(\frac{1 + K_i[I]}{\bar{K}K'_s}\right)^{1/2} \quad (8)$$

where $\bar{K} = k_1/(k_{-1} + k_2)$. In the second case, $a\alpha\bar{K}_i$ is equal to K_i. It is then found that

$$[S]_{opt} = \left[(1 + K_i[I])\{k_{-1} + k_2(1 + K_i[I])\}\frac{1}{k_1K'_s}\right]^{1/2} \quad (9)$$

In the third case the complex ESI does not react to form products; that is, a equals zero. Here

$$[S]_{opt} = \left\{\frac{1 + K_i[I]}{\bar{K}K'_s}\right\}^{1/2} \quad (10)$$

Equations 10 and 8 are seen to be identical.

The effect on this mechanism of addition of the inhibitor to the acetyl-enzyme can be determined by deriving the rate equation for the scheme shown in Fig. 3. The substrate again adds on to the Michaelis complex, but for the purposes of the present discussion addition of the inhibitor to the Michaelis complex can be neglected. The rate equation is found to be

$$v = \frac{k_2[E]_0\bar{K}[S]}{1 + K_i[I] + \bar{K}[S]\left\{1 + K'_s[S] + \frac{k_2}{k_3}\frac{(1 + \beta\bar{K}_i[I])}{(1 + a\beta\bar{K}_i[I])}\right\}} \quad (11)$$

The optimum substrate concentration is

$$[S]_{opt} = \left\{\frac{1 + K_i[I]}{\bar{K}K'_s}\right\}^{1/2} \quad (12)$$

The fact that this result is independent of the values of a and $\beta\bar{K}_i$ is particularly important from the standpoint of the present discussion.

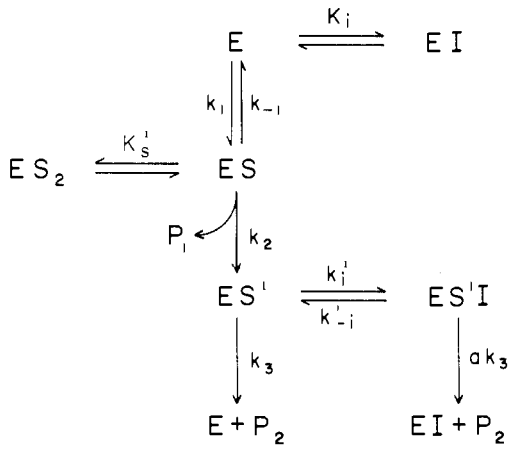

Fig. 3.—Another scheme for substrate inhibition, in which the inhibitor combines with E and ES' but the second substrate molecule combines with ES; the data are inconsistent with this scheme.

The conclusions resulting from the comparison of the scheme in Fig. 1 with those in Figs. 2 and 3 may now be summarized. Consideration of the scheme in Fig. 2 shows that, depending upon whether or not the inhibitor adds on to the Michaelis complex and upon whether or not the ESI complex can react further, the substrate optimum may be related to the first power or square root of the inhibitor concentration (equations 8, 9 and 10). Equation 12, derived on the basis of the scheme in Fig. 3, shows that whatever this relation is, it is not altered by addition of the inhibitor to the acetyl enzyme. This conclusion is independent of whether the acetyl enzyme–inhibitor complex is inactive or can react to form products. It is therefore possible to distinguish experimentally between substrate inhibition caused by addition to the acetyl enzyme from that caused by addition to a previously formed reaction intermediate (such as the Michaelis complex), provided that an inhibitor is available which is known to become attached to the acetyl enzyme and to block deacetylation.

Application to Acetylcholinesterase.—It has been shown that *cis*-2-dimethylaminocyclohexanol blocks deacetylation but does not block the acetylation process in the acetylcholine–acetylcholinesterase system. It also competes with the substrate for the free enzyme. The action of this inhibitor is thus of the apparent non-competitive type.[5] Other inhibitors, such as choline, exhibit simple competitive behavior.[8,9] If the scheme shown in Fig. 1 actually represents the behavior of the acetylcholinesterase system, the relation of substrate optimum to *cis*-2-dimethylaminocyclohexanol concentration should be that given in equation 6. For simple competitive inhibitors the relation is of the form of equation 5 or 6, depending upon whether or not the inhibitor attaches in a non-inhibiting manner to the site of substrate inhibition.

Several sets of results applying to the effect of competitive inhibitors on the acetylcholine–acetylcholinesterase system are available in the literature. The data of Augustinsson and Nachmansohn[10] for prostigmine and those of Augustinsson[11] for choline are plotted in Fig. 4. It is seen that the substrate optimum is related to the first power of the inhibitor concentration, in agreement with equation 5. A shift in substrate optimum at one concentration of eserine was demonstrated by Burgen.[12] According to equation 5 the ratio of the optima in the presence and absence of inhibitor is approximately equal to $1 + K_i[I]$. Use can be made of the value for the reciprocal of K_i (6.1×10^{-8}) determined by Augustinsson and Nachmansson[10] under what appear to be similar conditions; for the inhibitor concentration used, 2.62×10^{-7} M, the predicted ratio is approximately 5.30. The actual ratio is 5.15, in excellent agreement with equation 5.

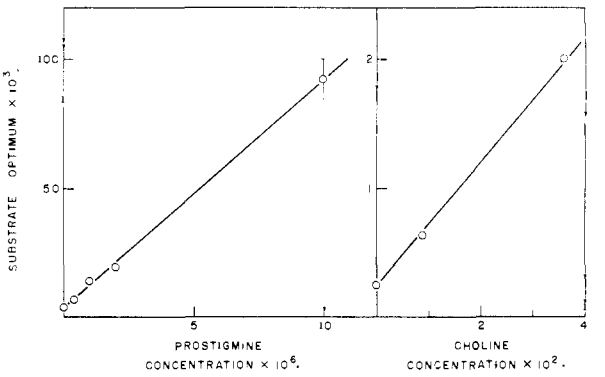

Fig. 4.—Plots of substrate concentration at the optimum against inhibitor concentration, for the acetylcholine hydrolysis; the prostigmine data are from Augustinsson and Nachmansohn,[10] those for choline from Augustinsson.[11]

The interpretation of these results is therefore that choline, prostigmine and eserine must become attached in a non-inhibitory manner to the site of substrate inhibition. The substrate and these inhibitors all contain a positive nitrogen atom. In the case of choline, Wilson[8] concluded that the hydroxyl group is not important in the binding, the principal attractive force being due to the trimethylammonium group. It follows that the site of substrate inhibition must contain an anionic group.

The critical test of the theory is therefore the behavior of the system in the presence of *cis*-2-dimethylaminocyclohexanol, since this inhibitor was shown to block deacetylation.[5] As noted above, the behavior with this inhibitor should be in agreement with equation 6.

Experimental

The general procedure and experimental materials have been described in Part I of this series.[5]

Results

The variation in the substrate optimum at several concentrations of *cis*-2-dimethylaminocyclohexanol is shown in Fig. 5. The solid line drawn from the substrate optimum in the absence of inhibitor and intersecting the other curves near the

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

(9) R. M. Krupka and K. J. Laidler, THIS JOURNAL, **83**, 1454 (1961).

(10) K. B. Augustinsson and D. Nachmansohn, *J. Biol. Chem.*, **179**, 543 (1949).

(11) K. B. Augustinsson, *Acta Physiol. Scand.*, **15**, Suppl. 2 (1948).

(12) A. S. V. Burgen, *Brit. J. Pharmacol.*, **4**, 219 (1949).

experimental maxima was calculated from equation 6. The broken line was calculated from equation 5. It is seen that the experimental curves have maxima which are in excellent agreement with the values calculated from equation 6.

Discussion

In general, substances containing a positively charged quaternary nitrogen atom inhibit acetylcholinesterase competitively.[8,13] These substances are bound to the anionic site in the enzyme surface, to which the substrate itself (acetylcholine) is normally attached. The inhibitors choline, prostigmine, eserine and *cis*-2-dimethylaminocyclohexanol all possess such a positive nitrogen atom. Work by Friess and Baldridge[7] on inhibitors which are functionally similar to *cis*-2-dimethylaminocyclohexanol has shown that the positive nitrogen of these compounds is indeed involved in binding to the enzyme. It may therefore be concluded that acetylcholine and all the inhibitors, including *cis*-2-dimethylaminocyclohexanol, become attached to the anionic site. Choline, prostigmine and eserine were also shown to become attached to the same site as the inhibiting molecule of substrate, and it was shown that this site must also be an anionic site. It follows that *cis*-2-dimethylaminocyclohexanol should also be able to add on to the latter site.

The experimental results have shown that the substrate optimum is related to the concentration of *cis*-2-dimethylaminocyclohexanol by a square root law (equation 6). It was shown that this relation is obtained only if (1) the inhibitor and excess substrate do not compete for the same site, or (2) they do compete for the same site, but both the substrate and the inhibitor block further reaction of the intermediate to which they are bound. The first explanation is excluded, and therefore both the inhibitor and substrate must block the reaction at the same stage in the reaction sequence. The inhibitor in question was shown to block deacetylation only[5]; it follows that it is this reaction which is inhibited by excess substrate. It then follows from the fact that the substrate inhibits deacetylation that the inhibitors eserine, prostigmine and choline must become attached to the acetyl enzyme without appreciably blocking deacetylation, since otherwise the first power dependence of substrate optimum on the inhibitor concentration would not be obtained (equations 2 and 5).

The scheme proposed (Fig. 1) is therefore supported by the experimental evidence in a number of ways. Thus the facts which have been brought out are:

(1) *cis*-2-Dimethylaminocyclohexanol competes with the substrate for the anionic site in the free enzyme; it is also bound to the anionic site in the acetyl-enzyme, and in this position it interacts with the esteratic site and blocks deacetylation.

(2) The substrate can also become attached to the anionic site in the acetyl-enzyme. It can then interact with the esteratic site, blocking deacetylation.

(3) Certain inhibitors such as choline, prostigmine and eserine compete with the substrate for the free enzyme. They also become attached to the acetyl-enzyme without blocking deacetylation.

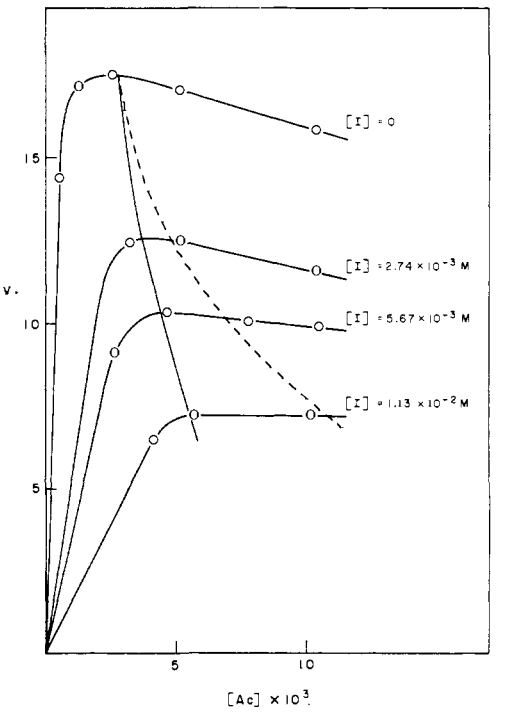

Fig. 5.—Plots of rate against acetylcholine concentration for various concentrations of *cis*-2-dimethylaminocyclohexanol. The full curve through the maxima is the theoretical curve corresponding to eq. 6; the dotted curve that corresponding to eq. (5). (The units of V are 10^{-10} mole sec.$^{-1}$)

The Effect of the k_2/k_3 Ratio on Substrate Inhibition.—It has been reported by Wilson[8] that although acetylcholinesterase is inhibited by high concentrations of acetylcholine, it is not inhibited by high concentrations of N-dimethylaminoethylacetate, a compound which closely resembles acetylcholine. Studies of Wilson and Cabib[4] have shown that the ratio of k_3 to k_2 is larger for dimethylaminoethylacetate than for acetylcholine. It is seen from equation 4 that the substrate optimum is proportional to the square root of the ratio k_3/k_2; the substrate optimum of the former substance would therefore occur at a higher value than that of acetylcholine. A possible explanation for the failure to detect substrate inhibition may be that sufficiently high concentrations of substrate were not tested.

The Second Anionic Site.—It is known that substances possessing two quaternary nitrogen atoms separated by a distance of approximately 14 Å, such as curare and stilbamidine, are extremely powerful inhibitors of acetylcholinesterase.[14] An explanation which has been given for this behavior[2] involves the postulate that there are two negative sites in each active center of the enzyme, separated by a distance of about 14 Å. The inhibitor is thought to attach itself simultaneously to these sites and to cover up the esteratic site in doing so. According to this explanation monoquaternary ions only inhibit appreciably when two of them are attached to the enzyme—one at each negative site. Inhibition by substrate occurs when substrate

(13) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **185**, 479 (1950).

(14) R. B. Barlow and H. R. Ing, *Nature*, **161**, 718 (1948),

molecules are bound to both anionic sites. The distances between each negative group and the esteratic site is thought to be about 7 Å.

The suggestion that the binding of two mono-quaternary ions is necessary for inhibition is ruled out since the reciprocal of the velocity is proportional to the concentration of the inhibitor rather than to the square of the concentration. The explanation of substrate inhibition is ruled out by the present experiments. Friess and Baldridge[7] concluded from inhibition studies with substituted cyclic compounds that the distance from the anionic site to the group in the esteratic site with which the electronegative substituent of the inhibitor interacts is not greater than 2.5 Å. Wilson and Quan[15] found that the distance between the anionic site and the basic group in the catalytic center was approximately 5 Å. These findings therefore appear to be in disagreement with the postulate of the second anionic site.

The explanation of the powerful inhibition shown by drugs such as stilbamidine may be twofold. It seems likely that the second cationic ammonium group interacts with a negative group on the enzyme surface, but this group may not be closely associated with the active center, either functionally or spatially. It was shown by Cavallito and Sandy[16] that among bis-quaternaries of equal hydrocarbon chain length there is often a marked increase in anticholinesterase activity associated with an increase in the lipophilicity of the substituents on the positive nitrogen atoms. These authors suggest that the increased inhibitory power associated with increased chain length may be due partly or wholly to increased lipophilicity rather than to interaction with two negative sites.

Neutralization of the Anionic Site.—Bergmann and Shimoni[17] showed that inhibition by the tetramethylammonium ion drops off rapidly below *p*H 7, suggesting that the anionic group, to which the inhibiting ion becomes attached, is neutralized. The apparent pK for the dissociation is 6.5. Since no negative groups which add on a proton at this *p*H are known in proteins, Bergmann and co-workers[18] suggested that the behavior was due to the interaction of the anionic group with an adjacent residue which was positively charged below *p*H 6.5. They suggested that the latter might be the basic group of the esteratic site, whose pK is 6.5. The difficulty with this explanation is that when the basic group accepts a proton, becoming positively charged, the enzyme is no longer able to catalyze the hydrolysis of the substrate. In order to explain the experimental observation the enzyme must hydrolyse the substrate but must be unable to complex with the inhibitor. It therefore appears necessary to postulate a second group adjacent to the anionic site which becomes positively charged below *p*H 6.5. This group may have no direct role in the enzymatic catalysis.

(15) I. B. Wilson and C. Quan, *Arch. Biochem. Biophys.*, **73**, 131 (1958).

(16) C. J. Cavallito and P. Sandy, *Biochem. Pharmacol.*, **2**, 233 (1959).

(17) F. Bergmann and A. Shimoni, *Biochim. Biophys. Acta*, **9**, 473 (1952).

(18) F. Bergmann, R. Segal, A. Shimoni and M. Wurzel, *Biochem. J.*, **63**, 684 (1956).

Structural Requirements for Substrate Inhibition.—It is probable that interaction of the substrate with the esteratic sites in the free and acyl enzymes is of a different nature; in the former a bond is formed between an electrophilic carbon atom and the basic group of the enzyme, while in the latter there is probably attraction between an electronegative grouping of the substrate and the acid group in the enzyme. In acetylcholine the required electronegative group may be the carbonyl oxygen atom.

Certain halogen-substituted esters which lack a quaternary nitrogen atom, such as isopropyl bromoacetate and ethyl chloroacetate, are hydrolyzed by acetylcholinesterase.[19] These substances also shown inhibition by excess substrate, and with them there is no question of attachment to an anionic site. A probable explanation of inhibition by these compounds is that the halogen atom present in the acyl group of the substrate is strongly electronegative and interacts with the acidic part of the esteratic site in the acyl enzyme. This suggestion is supported by the fact that certain substances containing a chlorine atom inhibit acetylcholinesterase in a non-competitive manner,[20] indicating that they block deacetylation.

The size and shape of the molecule may determine whether or not it can block deacetylation, for the presence of the acetyl group may prevent contact between a large inhibitor and the esteratic site. This is particularly true if the optimal distance between the latter and the anionic site is not greater than 2.5 Å, as shown by Friess and Baldridge.[7] This, together with the fact that the interactions with the esteratic site in the free and acyl enzymes appear to be of a different nature, may account for the much smaller size of the association constant for substrate inhibition as compared with substrate binding to the free enzyme ($\bar{K}$, of course, represents a minimum for the strength of binding of enzyme and substrate).

Acknowledgment.—Thanks are due to the Defence Research Board of Canada for their support of this work under Grant 9510-06.

Appendix

Further Discussion of the Theory.—The theory of substrate inhibition developed was shown to predict the shift in the substrate optimum in the presence of two classes of inhibitors. The theory can be checked further to see whether it predicts the change in the maximum velocity, as well as the general shape of the curves obtained as the substrate concentration is increased. When this is done, it is found that the theory is in complete agreement with the results with *cis*-2-dimethylaminocyclohexanol but that the behavior with choline and prostigmine is more complicated than the simple theory suggests.

The behavior with *cis*-2-dimethylaminocyclohexanol may be considered first. It can be shown by substituting equation 2 into 1 and putting α equal to zero and β equal to unity that the reciprocal of the maximum velocity is

$$\frac{1}{v_{max}} = \left(\frac{K_s}{k_2k_3\bar{K}}\right)^{1/2}\frac{1}{[E]_0}\left(\frac{k_3\bar{K}}{k_2K_s} + 2(1 + K_i[I])^{1/2} + \left(\frac{k_2\bar{K}}{k_3K_s}\right)^{1/2}(1 + K_i[I])\right) \quad (13)$$

(19) F. Bergmann and A. Shimoni, *ibid.*, **55**, 50 (1953).

(20) S. L. Friess, THIS JOURNAL, **79**, 3269 (1957).

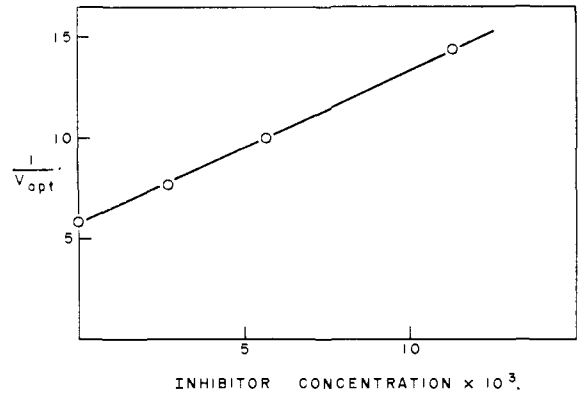

Fig. 6.—Plots of reciprocal of optimum velocity against concentration of *cis*-2-dimethylaminocyclohexanol. (The units of $1/V$opt are 10^8 sec. mole^{-1}.)

Substitution of the experimental value[21] for K_s and $\frac{k_2}{k_3}\bar{K}$, which is approximately equal to the experimental value of $\bar{K}$, shows that the factor $(k_2\bar{K}/k_3K_s)^{1/2}$ is approximately equal to 10. The term in the first power of $1 + K_i[I]$ in equation 13 will therefore predominate over that in the square root, and a plot of $\frac{1}{v_{max}}$ against the inhibitor concentration should be linear. This is shown to be the case in Fig. 6. It is also easily shown by substituting numerical values into equation 1 (with $a = 0$ and $\beta = 1$) and plotting the data graphically that the theory predicts curves of the shape found experimentally (Fig. 5).

In the work of Augustinsson[11] on cholinesterase plots of several general shapes were obtained. These are illustrated in Fig. 7. The plot for the enzyme from *Sepia* "liver" inhibited by clupeine (curve III) corresponds to equation 1. The latter predicts that at sufficiently high concentrations of substrate the rate is increased by the addition of the inhibitor; thus under these conditions equation 1 becomes

$$v = \frac{k_3[E]_0(1 + a\beta\bar{K}_i[I])}{K_s[S]} \qquad (14)$$

At higher substrate concentrations than are shown in curve III the rate should diminish.

The other plots however are more complicated. That for the enzyme from bovine erythrocytes in the presence of clupeine (curve V) shows an inhibition at very high substrate concentrations which tends to be independent of the inhibitor concentration. At somewhat lower substrate concentrations, however, the rate is increased in the presence of the inhibitor. In the absence of the inhibitor the rate decreases more slowly than the simple theory predicts (that is, with b equal to zero), as is shown by a comparison of curves I and II. Another example of such behavior may be the enzyme from *Helix* blood in the presence of choline (curve V). These curves are explained if b is greater than zero and if the substrate can interact with the esteratic site even when the inhibitor is attached to the anionic site. Such addition of the substrate to the acetyl-enzyme–inhibitor complex would then be non-competitive with respect to the inhibitor. The association constant for this addition would of course be smaller than that for the addition of the substrate to the acetyl-enzyme, so that at the substrate optimum little of the complex ES'IS would be formed. Another possible contributing factor is that, even if β is less than unity, the plot according to equation 2 ($[S]_{opt}$ against $[I]$) may appear to be linear, provided that β does not become very small. In this case the inhibitor is less effective in protecting the acetyl-enzyme against substrate inhibition.

With prostigmine and choline the rate in the presence of the inhibitor at no time exceeds that in its absence, and at very high substrate concentrations the two rates closely approach one another (curve VI). We have seen that, with these inhibitors, $\frac{1}{v}$ and $[S]_{opt}$ are linear functions of $[I]$. The explanation of this complex behavior may be similar to that suggested in the preceding paragraph. An additional factor is discussed below.

(21) I. B. Wilson, in W. D. McElroy and B. Glass, editors, "Symposium on the Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, Md., 1954, p. 642.

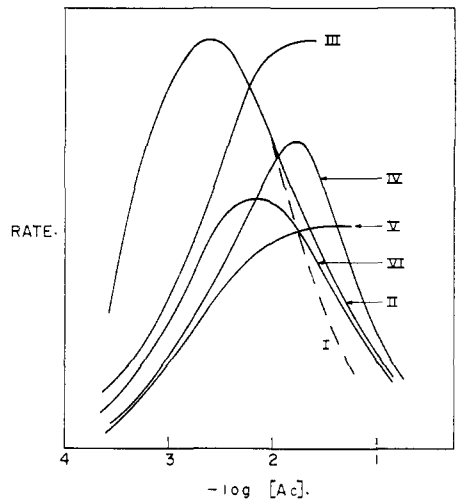

Fig. 7.—Plots of rate of acetylcholine hydrolysis against the logarithm of the substrate concentration. Curve I is the theoretical curve for no inhibitor, corresponding to the simple theory with $b = 0$; II is the experimental curve for bovine erythrocyte cholinesterase which deviates slightly at the higher concentrations. Curve III is for the enzyme from *Sepia* "liver" inhibited by clupeine; IV for bovine erythrocyte cholinesterase inhibited by clupeine; V for the enzyme from *Helix* blood inhibited by choline; VI for bovine erythrocyte inhibited by choline. (All data are from Augustinsson.[11])

The Effect of Irreversible Inhibitors on the Substrate Optimum.—The effect of diisopropylphosphorofluoridate (DFP) and tetraethylpyrophosphate (TEPP) on the substrate inhibition of the acetylcholine-acetylcholinesterase system was studied by Augustinsson and Nachmansohn.[10] It was found that the substrate optimum was not altered in the presence of the inhibitor, although the optimum velocity was reduced. In this system

$$E + I \xrightarrow{k_i} EI \qquad (15)$$

At the time that the experimental measurement is made, the amount of the enzyme-inhibitor complex can be considered to be constant. For simplicity we may consider the scheme of substrate inhibition where

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + \text{products} \qquad (16)$$

and

$$ES + S \rightleftarrows ES_2 \qquad (17)$$

The steady-state treatment of this scheme shows that

$$v = \frac{k_2([E]_0 - [EI])\bar{K}[S]}{1 + \bar{K}[S]\ (1 + K_s[S])} \qquad (18)$$

where $K = k_1/(k_{-1} + k_2)$ and $K_s = [ES_2]/[ES][S]$ The substrate optimum is

$$[S]_{opt} = \left\{\frac{1}{K\bar{K}_s}\right\}^{1/2} \qquad (19)$$

Equations 18 and 19 are in agreement with the experiments, since the substrate optimum is independent of the inhibitor concentration and the velocity is reduced in the presence of the inhibitor.

Although experiments reported by Burgen[12] and Augustinsson[11] showed that the substrate optimum was shifted greatly

in the presence of eserine, experiments carried out by Augustinsson and Nachmansohn[10] showed that the optimum was shifted only slightly with this inhibitor. The explanation for these seemingly contradictory results is probably that in the experiments of the latter workers the inhibitor was used under essentially irreversible conditions. This is possible because in the presence of the substrate a period of about 8 minutes is required before eserine and the enzyme equilibrate, forming the enzyme–eserine complex, while a longer period of 40 to 50 minutes is required for the dissociation of the latter.[12] The inhibition is therefore virtually irreversible if the enzyme and inhibitor are mixed before addition of the substrate, as was the case in the experiments of Augustinsson and Nachmansohn.[10]

In the experiments with prostigmine carried out by the latter workers,[10] the inhibitor and enzyme were incubated together for 1 hr. before addition of the substrate, as in the work with eserine. The behavior observed with prostigmine may therefore reflect both an effect of the type described by equation 2 and an irreversible inhibition producing a decrease in rate over the entire substrate range but resulting in no shift in substrate optimum. In this regard it is interesting to note that Wilson[22] used such a procedure of mixing prostigmine and the enzyme before adding the substrate in order to study the inhibition under non-competitive, that is essentially irreversible, conditions. Since a similar experimental procedure was used in the work with choline discussed above, it is concluded that an irreversible type of inhibition may contribute to the observed behavior of the system at high substrate concentrations.

(22) I. B. Wilson, *Biochim. Biophys. Acta*, **7**, 466 (1951).

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Molecular Mechanisms for Hydrolytic Enzyme Action. III. A General Mechanism for the Inhibition of Acetylcholinesterase

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General steady-state equations are derived for enzyme substrate–inhibitor systems in which reaction involves two intermediates, such as an addition (Michaelis) complex and an acyl enzyme. The inhibitor is assumed to combine with the free enzyme and the acyl enzyme, but not with the addition complex, and attachment of inhibitor to the acyl enzyme may or may not block deacetylation. It is shown that if the slow step is the transition from addition complex to acyl enzyme the inhibition is always competitive, and the same is always true if the inhibitor does not block deacylation. Non-competitive inhibition results if, and only if, the deacylation is the slow process and the inhibitor blocks deacylation. An experimental study of the inhibition of acetylcholinesterase action by choline, carbachol and eserine shows the behavior to be competitive even with acetylcholine as substrate, and the conclusion is that these inhibitors, unlike *cis*-2-dimethylaminocyclohexanol (Part II), do not block deacetylation. The structural requirements for the blocking of deacetylation are considered to be that the inhibitor contains in addition to its cationic center a center of high electron density; the molecule must also be sufficiently compact for the latter center to be able to interact with the acidic site on the enzyme without interfering with the acetyl group.

Introduction

It has been shown in the preceding papers[1] that substances containing a quaternary nitrogen atom can add on to the free and acetylated enzymes but not to the Michaelis complex. They can do so because the grouping in the enzyme to which they become attached, the anionic site, is free in the free and acetyl enzymes but is bound to the substrate in the Michaelis complex. If an inhibitor attached to the acetyl-enzyme can interact in a specific way with the esteratic site, deacetylation is blocked. For such interaction to occur the inhibitor must contain an appropriately placed electronegative function,[2] and it is believed that this is bound to the acid group in the esteratic site.[1a]

The effect of this behavior on inhibition caused by high concentrations of substrate was studied in part II.[1b] It was concluded that substrate inhibition results from addition of the substrate to the acetyl-enzyme rather than to the Michaelis complex. There is therefore now a need to develop the theory for cases of low substrate concentration and to decide if experimental data are in agreement with the theory under these conditions. The simplified reaction scheme, in which substrate inhibition is neglected, is shown in Fig. 1. As in the previous formulation[1b] the inhibitor adds on to the free and acetyl enzymes (E and ES′, respectively). Addition to the latter may or may not result in inhibition of deacetylation; that is, the factor a may be equal to zero or unity or some intermediate value.

Application of the steady-state treatment to this scheme gives rise to

$$v = \frac{k_2[\mathrm{E}]_0\bar{K}[\mathrm{S}]}{1 + K_i[\mathrm{I}] + \bar{K}[\mathrm{S}]\left\{1 + \frac{k_2(1 + \beta\bar{K}_i[\mathrm{I}])}{k_3(1 + a\beta\bar{K}_i[\mathrm{I}])}\right\}} \quad (1)$$

where $\beta\bar{K}_i = k_i'/(k'_{-i} + ak_3)$ and $\bar{K} = k_1/(k_{-1} + k_2)$.

If k_2 is much smaller than k_3, equation 1 describes the case of simple competitive inhibition, since the term

$$\frac{k_2(1 + \beta\bar{K}_i[\mathrm{I}]}{k_3(1 + a\beta\bar{K}_i[\mathrm{I}]}$$

is negligible under these conditions.

If k_2 is equal to or larger than k_3, equation 1 may lead to a non-competitive form of inhibition. If a is equal to unity, however, simple competitive inhibition results under any circumstances. Thus if $a = 1$, equation 1 becomes

$$v = \frac{k_2[\mathrm{E}]_0\bar{K}[\mathrm{S}]}{1 + K_i[\mathrm{I}] + \bar{K}[\mathrm{S}]\left(1 + \frac{k_2}{k_3}\right)} \quad (2)$$

This is the equation for pure competitive inhibition.

Non-competitive inhibition results if $a = 0$. In this case equation 1 becomes

$$v = \frac{k_2[\mathrm{E}]_0\bar{K}[\mathrm{S}]}{1 + K_i[\mathrm{I}] + \bar{K}[\mathrm{S}]\left\{1 + \frac{k_2}{k_3}(1 + \beta\bar{K}_i[\mathrm{I}])\right\}} \quad (3)$$

(1) R. M. Krupka and K. J. Laidler, (a) THIS JOURNAL, **83**, 1445 (1961); (b) *ibid.*, **83**, 1448 (1961).

(2) S. L. Friess and W. J. McCarville, *ibid.*, **76**, 1363 (1954).