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Molecular Mechanisms for Hydrolytic Enzyme Action. I. Apparent Non-competitive Inhibition, with Special Reference to Acetylcholinesterase

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General rate equations are developed for the case in which an inhibitor may combine with the free enzyme, the enzyme–substrate complex, and with a subsequently formed enzyme–substrate intermediate. It is shown how the equations may be used to study the nature of the inhibitor binding. An experimental investigation of the inhibition of acetylcholinesterase by *cis*-2-dimethylaminocyclohexanol, using two substrates, showed that the inhibitor combines with the acetyl enzyme, blocking deacetylation, but not with the enzyme–substrate complex. It is shown that when this situation arises the inhibition will be of the mixed competitive and non-competitive type, even though the inhibitor and substrate compete for the same sites on the enzyme surface.

Introduction

According to conventional ideas of the action of enzyme inhibitors,[1] pure competitive inhibition results when an inhibitor combines with the same enzyme site as does the substrate, thereby preventing the formation of a complex between enzyme and substrate. Non-competitive inhibition results when the inhibitor and substrate may simultaneously combine with the enzyme, only the enzyme–substrate complex which is free of combined inhibitor being able to form products. If the equilibrium constants for the combination of inhibitor with free enzyme and enzyme-substrate complex are equal, "simple" or "pure" non-competitive inhibition is observed. If, on the other hand, these equilibrium constants are different, a more complicated inhibition law is observed and the inhibition is sometimes described as "mixed"; the inhibition has some of the characteristics of competitive inhibition and some of non-competitive. Previous formulations of the inhibition laws have applied to the situation in which enzyme and substrate form a single addition complex, which gives rise to products in a single stage. Evidence is now accumulating, however, that in many enzymes there are two enzyme–substrate intermediates; in the hydrolytic enzymes, for example, there is first an addition (Michaelis) complex and then an acylated enzyme.[2] There is therefore a need for the formulation of the inhibition laws for this type of situation and this has been met in the present paper. It will be shown that certain complications arise: for example, a purely competitive mechanism may lead to a law of the non-competitive or mixed type. The present treatment is closely related to our discussion of *p*H effects in systems involving two intermediates.[3]

General Rate Equations.—Figure 1 shows a reaction scheme involving two enzyme–substrate intermediates; the first, ES, may be regarded as the addition complex and the second, ES′, the acyl enzyme. The free enzyme and the two intermediates may combine with the inhibitor, the association constants being respectively K_i, αK_i and βK_i, α and β being positive numbers. Application of the steady-state treatment to this system gives the equation

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

where $\bar{K}$ is equal $k_1/(k_{-1} + k_2)$. This equation may be written as

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

so that a plot of $1/v$ against the inhibitor concentration for various substrate concentrations will give straight lines with intercepts and slopes that in general are functions of the substrate concentration. A plot of the slopes of such lines against the reciprocal of the substrate concentration will give a straight line having an intercept on the slope axis equal to

$$\left\{\frac{\alpha + \frac{k_2}{k_3}\beta}{k_2[E]_0}\right\}K_i \quad (3)$$

Acetylcholinesterase Inhibition.—One particular system to which these equations appear to apply is the acetylcholinesterase system. The catalytic center of this enzyme consists of an esteratic site which interacts with the ester bond of the substrate and an adjacent negatively charged anionic site.[4] Acetylcholinesterase is inhibited by compounds which, like the substrate acetylcholine, contain a positively charged quaternary nitrogen atom which becomes attached to the anionic site. Such inhibitors compete with the substrate for the free enzyme and in addition might be expected to interfere with the further reaction of the Michaelis complex or the acetyl enzyme. Certain of the substances of this type were found by Friess and co-workers to inhibit in a non-competitive or in a mixed fashion.[5] This behavior would be quite unexpected if only one enzyme-substrate intermediate was taken into account but may be interpreted readily in terms of the scheme discussed above. It is possible to decide whether the inhibitor combines with the Mich-

(1) K. J. Laidler, "The Chemical Kinetics of Enzyme Action," The Clarendon Press, Oxford, 1958, Chap. 3.

(2) (a) A. K. Balls and F. L. Aldrich, *Proc. Natl. Acad. Sci. U. S.*, **41**, 190 (1955); (b) H. Gutfreund and J. M. Sturtevant, *Biochem. J.* **63**, 656 (1956); (c) I. B. Wilson and E. Cabib, THIS JOURNAL, **78**, 202 (1956).

(3) R. M. Krupka and K. J. Laidler, *Trans. Faraday Soc.*, **56**, 1467, 1477 (1960).

(4) D. Nachmansohn and I. B. Wilson, *Advances in Enzymol.*, **12**, 259 (1951).

(5) (a) S. L. Friess, THIS JOURNAL, **79**, 3269 (1957); (b) D. S. Masterson, S. L. Friess and B. Witkop, *ibid.*, **80**, 5687 (1958); (c) S. L. Friess, E. R. Whitcomb, R. C. Durant and L. J. Reber, *Arch. Biochem. Biophys.*, **85**, 426 (1959).

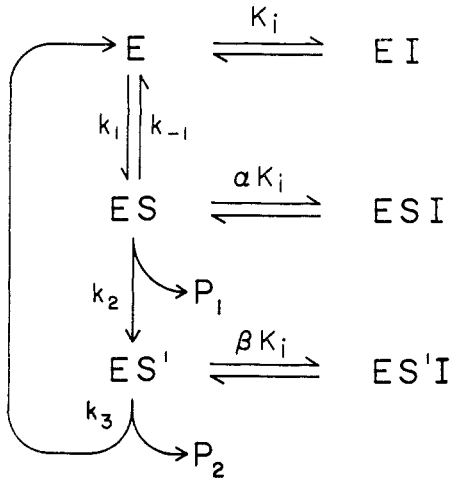

Fig. 1.—General reaction scheme for an inhibited system involving two enzyme–substrate intermediates.

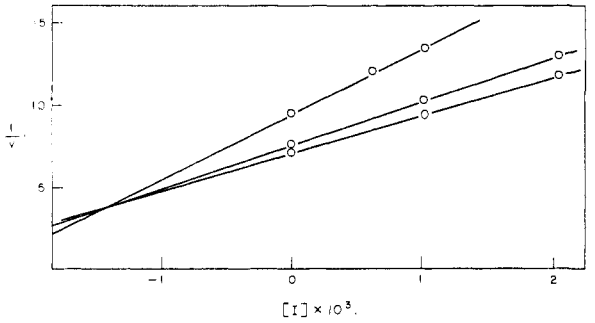

Fig. 2.—Plots of the reciprocal of the velocity constant against the concentration of *cis*-2-dimethylaminocyclohexanol, for the hydrolysis of acetylcholine; the units of $1/v$ are 10^8 sec. mole.$^{-1}$

aelis complex, the acyl enzyme or both, provided that inhibition of the hydrolysis of different substrates may be studied. The most convenient procedure is to choose substrates for which the relative values of k_2 and k_3 are quite different. If k_2 is very much larger than k_3 the intercept 3 reduces to $\beta K_i/k_3[E]_0$. A non-zero value of this intercept therefore indicates that the inhibitor combines with the acyl enzyme, *i.e.*, that the constant β is not zero. If, on the other hand, a substrate is chosen for which k_3 is much greater than k_2 the intercept is $\alpha K_i/k_2[E]_0$ and a positive value of this indicates combination of the inhibitor with the Michaelis complex.

It is known from the work of Wilson and Cabib[2c] that in the hydrolysis of acetylcholine $k_2 > k_3$, while in that of methylaminoethylacetate $k_3 > k_2$. These substances therefore provide a means for testing the present theory and in particular for seeing whether the inhibitor combines with the acyl enzyme, the Michaelis complex or both. Since it has been found by Friess[5a] that *cis*-2-dimethylaminocyclohexanol shows mixed non-competitive and competitive inhibition with acetylcholine as substrate, our experiments have been carried out using this substance.

Experimental

Materials.—The enzyme and substrates were the same as those used in previous work in these Laboratories[3]; the enzyme had been prepared from bovine erythrocytes.

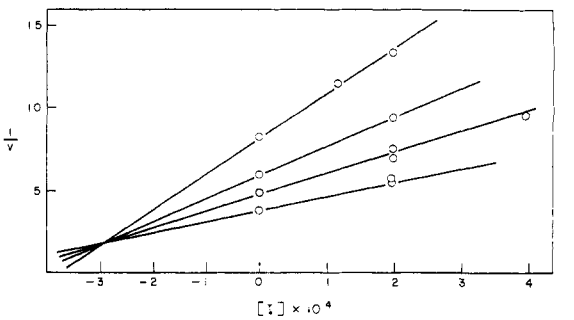

Fig. 3.—Plots of the reciprocal of the velocity constant against the concentration of *cis*-2-dimethylaminocyclohexanol, for the hydrolysis of methylaminoethyl acetate; the units of $1/v$ are 10^8 sec. mole^{-1}.

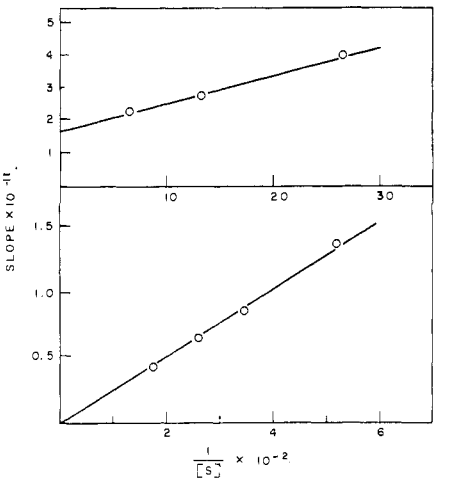

Fig. 4.—Plots of the slopes of the lines in Figs. 2 and 3, against the reciprocal of the substrate concentration; the upper figure is for acetylcholine, the lower for N-methylaminoethyl acetate.

The *cis*-2-dimethylaminocyclohexanol was a pure specimen kindly provided by Dr. S. L. Friess.

Kinetic Procedure.—The rates were measured using the automatic titration method previously described. The work was all done at 20.0°, using 15 ml. of the reaction mixture which was 0.04 *M* in $MgCl_2$ and 0.1 *M* in NaCl. The mixture of salts, substrate and inhibitor, in a volume of 14 ml., was allowed to come to equilibrium with respect to CO_2 concentration and temperature, and the reaction was begun by adding one ml. of enzyme solution to this mixture.

Results

The inhibition of the hydrolyses of both substrates by *cis*-2-dimethylaminocyclohexanol was studied at *p*H 6.75. Plots of the reciprocal of the velocity against inhibitor concentration (*cf.* equation [2]) are shown in Figs. 2 and 3. As shown by Dixon[6] for the case of the single enzyme-substrate intermediate, the intersection point of the plots of Fig. 2 provides a measure of K_i, the association constant between the inhibitor and the free enzyme, since at the intersection point $1/K_i = -[I]$.

It was seen above that the plots of the slopes of plots of $1/v$ against [I] against the reciprocal of the substrate concentration provide information about the inhibitor binding. Such plots are shown in Fig. 4. The intercept on the slope axis in the case of the acetylcholine inhibition is seen to have a non-zero

(6) M. Dixon, *Biochem. J.*, **55**, 170 (1953).

value, but the value is zero for methylaminoethyl acetate. On the basis of the theory the conclusion from this is that the inhibitor combines with the free enzyme and the acetyl enzyme but not with the Michaelis complex. The inhibition constants are found to be

K_i (acetylcholine hydrolysis) $= 7.1 \times 10^2$
K_s (MAEA hydrolysis) $= 9.7 \times 10^2$
βK_i (acetylcholine hydrolysis) $= 2.4 \times 10^2$

The value of βK_i was calculated using a value of k_2/k_3 equal to 6 with acetylcholine.[2c] The analysis of experiments in the absence of the inhibitor gives a value for V_{max} which is equal to $k_2k_3[E]_0/(k_2 + k_3)$, and it can be seen that $k_3[E]_0$ becomes equal to this if k_2 is much larger than k_3. When $k_2/k_3 = 6$, the ratio of the experimental value of V_{max} to $k_3[E]_0$ is 6/7.

Discussion

The main conclusion of the present work is that, in spite of the mixed type of inhibition found by Friess,[5a] the inhibitor *cis*-2-dimethylaminocyclohexanol does compete with the substrate as far as the formation of the Michaelis complex is concerned. This is consistent with the view that when the addition compounds are formed the inhibitor and substrate combine with the enzyme at the same site. Undoubtedly the anionic site participates in this combination. In the acetyl enzyme the anionic site is certainly free, while in the Michaelis complex it is not. Inhibitors may therefore become attached to the acetyl enzyme, and as a result there may be in some cases a non-competitive or mixed type of inhibition.

These conclusions may be compared with those of our previous work on the *p*H dependence of the action of acetylcholinesterase[3] in which it was shown that the basic and acidic groups in the catalytic center ionize in the free and acetyl enzymes. In the Michaelis complex the basic group is unable to ionize, and our interpretation of work on the DFP inhibition of the action of chymotrypsin suggested that the acidic group is also unable to ionize in the Michaelis complex.

It was concluded from studies on certain inhibitors by Friess and McCarville[7] that the requirement for strong binding to the enzyme is a polymethylated, charged nitrogen atom and a properly situated locus of high electron density. The former group would attach to the anionic site of the enzyme surface and the latter to the esteratic site. We have seen that in the free and acetyl enzymes both the basic and acidic groups are free. Since the basic group is an electron donor, it cannot be the site of inhibitor attachment. The acid group, on the other hand, could form a hydrogen bond with an electronegative grouping and may therefore be the group to which the inhibitor becomes attached. According to Friess and Baldridge,[8] the most effective inhibitors of the type which they studied have a separation of not more than 2.5 Å. between the positive nitrogen atom and the electronegative site. Wilson and Quan[9] conducted a similar study with a different type of inhibitor, namely, (hydroxyphenyl)-trimethylammonium derivatives. These compounds are believed to become attached to both the anionic site and the basic group, and it was concluded that the distance between these must be about 5 Å. If the distance of 2.5 Å. found by Friess and co-workers with their inhibitors is actually a measure of the distance between the anionic site and the acidic group in the enzyme surface, then this result, together with that of Wilson and Quan,[9] gives an indication of the geometry of the catalytic center, suggesting that the basic group is farther from the anionic site than is the acidic group. That this is a reasonable conclusion is shown by the following considerations. The basic group probably interacts with the carbonyl carbon atom of the substrate, and the acidic group may donate a proton to the alcohol portion of the substrate as the ester bond is split. The basic and acidic groups should therefore lie adjacent to the carbonyl carbon and ester oxygen atoms, respectively, of the substrate when the latter is bound to the enzyme in the Michaelis complex. It follows that the relative separations of the basic and acidic groups from the anionic site should reflect the separations of the carbonyl carbon and ester oxygen atoms from the trimethylammonium group in the substrate. In the latter the carbonyl carbon atom would expectedly be farther from the positive nitrogen atom than is the ester oxygen atom. The basic group should therefore be farther from the anionic site than the acidic group, in agreement with the interpretation of the work of Friess and Baldridge[8] and Wilson and Quan[9] which has been given.

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(7) S. L. Friess and W. J. McCarville, THIS JOURNAL, **76**, 1363 (1954).

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

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