down of the chymotrypsin acetyl-L-tyrosine intermediate is rate limiting (Dixon and Neurath, 1957; Hofstee, 1959, p. 187). If true, this would prohibit a direct comparison between the compound giving the optimum in substrate activity and the inhibitor giving optimum activity for these series of compounds.

The present results do show, however, that although a direct and detailed comparison of the effect of structure on substrate and on inhibitor activity must await further information, one can successfully use the expectation of a relation between them as a general guide in the search for more specific organophosphorus inhibitors. The results also make clear that in this search it will not be sufficient to find a structure which increases activity toward a given enzyme, but, in addition, the molecule will have to contain structural features which decrease reactivity toward other enzymes. This is given point by the demonstration that although the changes in length of the carbon chain of the alkylphosphonates had a distinct effect on the inhibitory activity toward chymotrypsin and trypsin, the inhibitory activity against acetyl cholinesterase was high with all of these compounds and was relatively little affected by these changes in structure. As a corollary of this, all of the compounds studied here have a high acute mammalian toxicity.

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The Mechanism of Action of Acetylcholinesterase: Substrate Inhibition and the Binding of Inhibitors

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The effects of the cationic inhibitors, tetramethylammonium and choline, on the inhibition of acetylcholinesterase by its substrate have been investigated. All the experimental results can be explained by a mechanism involving the formation of (*a*) an unreactive complex between the acetyl enzyme and the substrate and (*b*) a complex, at least partially reactive, between the acetyl enzyme and the inhibitor. This mechanism predicts a small noncompetitive component in inhibition by such cationic substances. It also predicts that plots of the reaction velocity against the substrate concentration at various concentrations of an inhibitor should form a family of curves having certain characteristic features with regard to (1) the optimum substrate concentration, (2) the reaction velocity at the optimum substrate concentration, and (3) the ability or inability of the inhibitor to increase the reaction rate at substrate concentrations above the optimum. The family of curves can be considered to fall into one of several broad classes, the behavior observed depending on the particular enzyme, substrate, inhibitor, and experimental conditions. It is shown that the experimental observations of this study and others conform to the predictions. In contrast to this behavior, the inhibition by *cis*-2-dimethylaminocyclohexanol, which contains a large noncompetitive component, can only be explained if the acetyl enzyme-inhibitor complex is unreactive.

It has long been known that the rate of the acetylcholinesterase-catalyzed hydrolysis of acetylcholine declines at high concentrations of the substrate. Recently it was shown that this substrate inhibition is probably due to the formation of a binary complex between a molecule of acetylcholine and the acetyl enzyme (Krupka and Laidler, 1961). The latter is a reaction intermediate derived from the Michaelis complex; it is formed when the acetyl group of the substrate is transferred from choline to a group at the active center of the enzyme (Wilson, 1960). Accordingly, the hydrolysis of the substrate may be represented as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + A$$
$$\qquad\qquad\qquad\qquad\quad \underset{C}{+}$$
$$EA + S \rightleftharpoons EAS$$

where E is the enzyme, S acetylcholine, ES the Michaelis complex, and EA the acetyl enzyme. C

and *A* are the reaction products, choline and acetic acid, respectively. *EAS* is the complex formed between the acetyl enzyme and the substrate, which does not react further.

This mechanism for substrate inhibition is supported by the behavior of the system in the presence of substances containing a positively charged quaternary nitrogen atom. Such substances are attracted to the negatively charged "anionic site" in the active center of acetylcholinesterase, which also attracts the quaternary nitrogen atom of acetylcholine. Consequently they compete with the substrate for attachment to the active center, and in this sense they are competitive inhibitors. In the Michaelis complex, the substrate is held by an electrostatic bond to the anionic site. Probably because of this neither the quaternary nitrogen inhibitors, nor a second molecule of substrate, appears to be bound to the Michaelis complex. However, in the acetyl enzyme the anionic site is free, the choline portion of the substrate having been split off in the formation of this intermediate. A substrate molecule may therefore become bound to the acetyl enzyme and may then block deacetylation (k_3). Quaternary nitrogen inhibitors are also bound to the acetyl enzyme. Although some of these block deacetylation to a large extent and so inhibit in a mixed competitive and noncompetitive manner (type I), others block deacetylation to a much smaller extent and so inhibit in an almost purely competitive manner (type II). Substances of the first type have an electronegative locus at a particular position relative to the quaternary nitrogen atom (Friess and McCarville, 1954). The inhibition may therefore be due to a specific interaction with a group in the enzyme which, unlike the anionic site, plays an essential role in deacetylation. This essential group may be the "acidic group" at the active center.

It was originally thought that type II inhibitors, such as choline, are purely competitive (Krupka and Laidler, 1961), but independent experiments by Wilson and Alexander (1962) and in this laboratory have revealed a small noncompetitive component in their inhibitions. The question therefore arises whether the actions of the types I and II inhibitors are in fact fundamentally different. The argument previously advanced depended partly upon experimental measurements of the optimum substrate concentration. Unfortunately it is difficult to measure this quantity accurately, and it is therefore desirable to test the idea in some other way. To this end a general treatment is given of the reaction scheme already proposed, and the experimental predictions that result from several mechanistic assumptions are considered. It is found that measurements of the optimum velocity can be used to test the theory, and these can be determined with sufficient accuracy for our purposes. To obtain appropriate data the system was studied with two simple quaternary nitrogen inhibitors, tetramethylammonium and choline ions. It is shown that the experiments cannot be explained if the complex formed between the acetyl enzyme and the inhibitor is totally unreactive, but can be explained if this complex is either partially or completely reactive. If it is completely reactive, the noncompetitive component of the inhibition would be due to a rate-limiting desorption of the inhibitor from the enzyme. The relative importance of these two mechanisms cannot be decided from the present experiments.

The present work also extends the previous treatment in accounting in a simple way for the observations on cholinesterase systems made by Augustinsson (1948). Augustinsson observed that in some systems the inhibitor lowers the reaction rate at all substrate concentrations, and in others it increases the rate at very high substrate concentrations. In a few cases the optimum velocity in the presence of the inhibitor is as high as that in its absence.

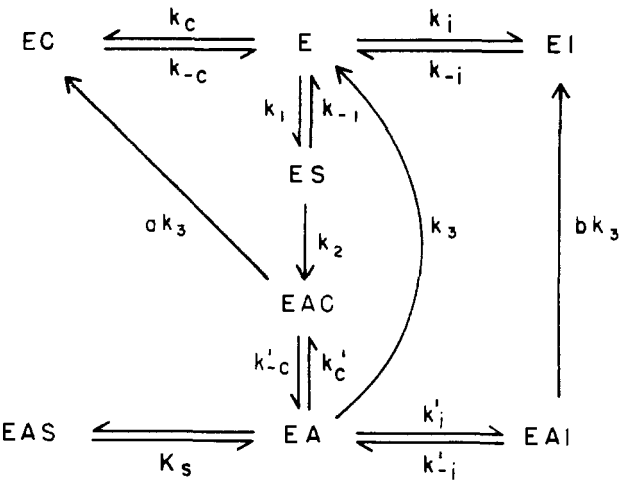

FIG. 1.—Reaction scheme for substrate inhibition and inhibitor binding: both the substrate and the inhibitor may become bound to the free enzyme (*E*) or the acetyl enzyme (*EA*). The complex of the acetyl enzyme with the inhibitor is reactive, but that with the substrate is unreactive (see text).

STEADY STATE THEORY

A reaction scheme for the hydrolysis of acetylcholine in the presence of inhibitory concentrations of the substrate and a quaternary nitrogen inhibitor other than choline is shown in Figure 1. This scheme is essentially the same as the one proposed previously (Krupka and Laidler, 1961), since the substrate (*S*) and inhibitor (*I*) compete for attachment at the anionic site, not only in the free enzyme (*E*) but also in the acetyl enzyme (*EA*). Once attached to the acetyl enzyme, the substrate blocks deacetylation, but the inhibitor does not necessarily do so. According to this mechanism, the Michaelis complex (*ES*) first reacts to form an acetyl enzyme in which the choline portion of the substrate is still bound at the anionic site (*EAC*). The latter complex may undergo deacetylation, giving the enzyme-choline complex (*EC*) and acetic acid, or alternatively the choline molecule may separate, leaving the acetyl enzyme free. The acetyl enzyme may then add on a molecule of acetylcholine, forming *EAS*, or a molecule of the inhibitor, forming *EAI*. In the general case, the deacetylations of *EA*, *EAC*, and *EAI* are governed by the rate constants k_3, ak_3, and bk_3 respectively. Simplifying assumptions are that *EAS* is completely unreactive, that the formation of *E* from *EC* is irreversible (since no choline is present at the beginning of the reaction), and that the acetylation reaction (k_2) is also irreversible.

The velocity of the reaction is found to be

$$v = \frac{k_2[E]_0\bar{K}[S]}{1 + K_i[I] + \bar{K}[S]\left\{p + \dfrac{q\bar{K}'_i[I] + rKs[S]}{1 + \bar{K}'_i[I]}\right\}} \qquad (1)$$

where

$\bar{K} = k_1/(k_{-1} + k_2)$, $K_i = k_i/k_{-i}$, $\bar{K}'_i = bk'_i/(k'_{-i} + bk_3)$,
$p = 1 + k_2/k_3 + \{k_2/(k'_{-c} + ak_3)\}\{(ak_3/k_{-c}) + 1 - a\}$,
$q = \{k_2k'_{-c}/k_3(k'_{-c} + ak_3)\}\{k_3/k_{-i} + 1/b - 1\}$ and
$r = k_2k'_{-c}/k_3(k'_{-c} + ak_3)$.

For the special case of choline inhibition, the concentration of I is put equal to zero, and the additions of choline to E and EA are taken into account. The equation for the reaction velocity is then found to be of the same form as (1).

We should now consider what experimental prediction this equation makes. By differentiating it with respect to $[S]$ and setting the differential equal to zero, it is found that the optimum velocity occurs when the substrate concentration is

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

If $K_i \simeq \bar{K}'_i$, this becomes

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

Equation (3) predicts a linear relationship between the substrate optimum and the inhibitor concentration. However, it can be shown that equation (2) leads to a nearly linear relationship over a certain range of inhibitor concentrations provided that K_i and $\bar{K}'_i$ do not differ by a factor of more than about 10.

The expression for the optimum velocity is obtained by substituting (3) into (1):

$$v_{opt} = k_2[E]_0 \Big/ \left\{p + 2(rK_s/\bar{K})^{1/2} + \frac{q\bar{K}'_i[I]}{1 + \bar{K}'_i[I]}\right\} \quad (4)$$

When

$$[I] = 0,\ v_{opt} = v^0_{opt} = k_2[E]_0/\{p + 2(rK_s/\bar{K})^{1/2}\} \quad (5)$$

Equations (4) and (5) show that the reaction rate at the optimum is lower in the presence of the inhibitor than in its absence. It follows from (4) that

$$\frac{1}{v_{opt}} - \frac{1}{v^0_{opt}} = \frac{q\bar{K}'_i[I]}{k_2E_0(1 + \bar{K}'_i[I])} \quad (6)$$

and

$$\frac{v^0_{opt}\, v_{opt}}{v^0_{opt} - v_{opt}} = \frac{k_2[E]_0}{q}\left\{1 + \frac{1}{\bar{K}'_i[I]}\right\} \quad (7)$$

Equation (7) has been derived for the condition that $K_i = \bar{K}'_i$. When this condition does not hold, it is found that

$$v_{opt} = k_2[E]_0 \Big/ \left\{p + \frac{q\bar{K}'_i[I]}{1 + \bar{K}'_i[I]} + 2\left(\frac{rK_s}{\bar{K}}\right)^{1/2}\left(\frac{1 + K_i[I]}{1 + \bar{K}'_i[I]}\right)^{1/2}\right\} \quad (8)$$

and

$$\frac{v^0_{opt}\, v_{opt}}{v^0_{opt} - v_{opt}} = k_2[E]_0 \Big/ \left\{\frac{q\bar{K}'_i[I]}{1 + \bar{K}'_i[I]} + 2\left(\frac{rK_s}{\bar{K}}\right)^{1/2}\left[\left(\frac{1 + K_i[I]}{1 + \bar{K}'_i[I]}\right)^{1/2} - 1\right]\right\} \quad (9)$$

Equation (9) reduces to the form of (7) when the second term in the denominator is small compared to the first. The relative sizes of these terms depends on the relative sizes of q and $2(rK_s/\bar{K})^{1/2}$, and of K_i and $\bar{K}'_i$. Furthermore, the second term is small when the concentration of the inhibitor is low, but becomes more important as the inhibitor concentration is raised. Thus although equation (7) predicts a linear relationship between $v^0_{opt}\, v_{opt}/(v^0_{opt} - v_{opt})$ and $1/[I]$, equation (9) predicts a deviation from linearity at high concentrations of the inhibitor.

In interpreting the experimental results, the following points may be noted. If the plot of $v^0_{opt}\, v_{opt}/(v^0_{opt}\, v_{opt})$ against $1/[I]$ has a positive slope and a zero intercept, then $q\bar{K}'_i$ has a positive value but $\bar{K}'_i$ is zero. These values result if $b = 0$ and if k'_i/k'_{-i} is positive. In this case $1/v_{opt}$ should be linearly related to $[I]$. If both the slope and intercept of the $v^0_{opt}\, v_{opt}/(v^0_{opt}\, v_{opt})$ plot are positive, then both $q\bar{K}'_i$ and $\bar{K}'_i$ have positive values, as do b and k'_i/k'_{-i}. If v_{opt} is independent of $[I]$, $q\bar{K}'_i = 0$ and $K_i = \bar{K}'_i$; q approaches zero when $b = 1$ and $k_{-i} \gg k_3$.

At low substrate concentrations, where substrate inhibition is negligible ($rK_s[S] < < p$), equation (1) gives rise to the following expression:

$$\frac{1}{v} = \frac{1}{k_2[E]_0}\left\{p + \frac{q\bar{K}'_i[I]}{1 + \bar{K}'_i[I]} + \frac{1 + K_i[I]}{\bar{K}[S]}\right\} \quad (10)$$

Hence a plot of $1/v$ against $1/[S]$ should be linear, but the $1/v$ intercept should vary with $[I]$. The extent of this variation depends on the values of q and $\bar{K}'_i$. The quantity $1 + K_i[I]$ is calculated from the $1/v$ against $1/[S]$ plots by dividing the slopes of the lines for the inhibited reaction by the slope of the line for the uninhibited reaction.

Experimental Methods

Initial rates of acetylcholine hydrolysis were measured by the electrometric titration method with use of a recording *p*H stat manufactured by Radiometer, Copenhagan. The work was done at 25° with 10 ml of the reaction mixture containing 0.04 M $MgCl_2$ and 0.1 M NaCl, in addition to the enzyme and substrate. Before addition of the enzyme, the mixture was flushed with CO_2-free air, and during the course of the reaction a stream of CO_2-free air was passed over the surface of the solution. These precautions prevent changes in *p*H due to changes in the CO_2 concentration of the reaction mixture.

The purified enzyme had been prepared from bovine erythrocytes and was supplied by Nutritional Biochemicals Corp.

Experimental Results

The dependence of the rate of acetylcholine hydrolysis upon the substrate concentration in the presence of either tetramethylammonium or choline is shown in Figures 2A and B respectively. In Figures 3A and B, a function of the optimum velocity ($v^0_{opt}\, v_{opt}/[v^0_{opt} - v_{opt}]$) is plotted against the reciprocal of the inhibitor concentration. Figures 4A and B show plots of the reciprocal of the velocity against the reciprocal of the substrate concentration for inhibition by tetramethylammonium and choline respectively. In Figure 4 the substrate concentrations (up to 4×10^{-3} M) are in the range where substrate inhibition is negligible. The following substrate and inhibitor constants were calculated from the plots in Figures 3 and 4: acetylcholine, $\bar{K} = 2.35 \times 10^3\ M^{-1}$; tetramethylammonium, $K_i = 210\ M^{-1}$, $\bar{K}'_i = 25\ M^{-1}$; choline, $K_i = 660\ M^{-1}$; $\bar{K}'_i = 50\ M^{-1}$.

Discussion

The effects of tetramethylammonium and choline ions upon the rate of acetylcholine hydrolysis appear to be entirely in accord with equation (1). The plots of $v^0_{opt}\, v_{opt}/(v^0_{opt} - v_{opt})$ against $1/[I]$ (Fig. 3) are approximately linear, in agreement with equations (7) and (9), and the intercepts have positive values. The plots of $1/v$ against $1/[I]$ (Fig. 4) are linear, and

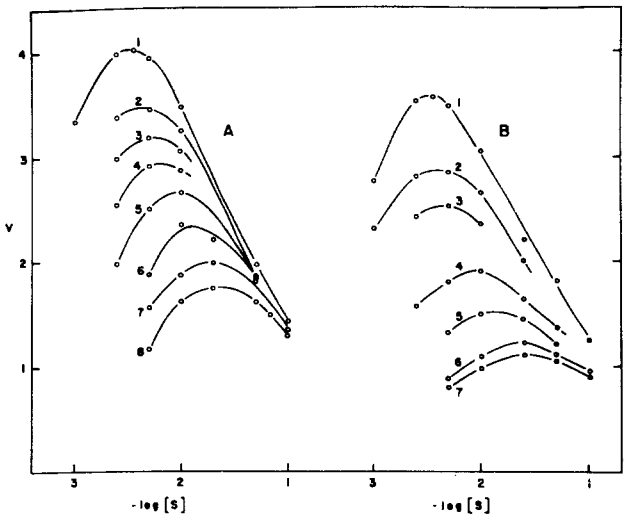

FIG. 2.—The rate of acetylcholine hydrolysis as a function of the negative logarithm of the substrate concentration (M), in the presence of various concentrations of a cationic inhibitor. In *A* the inhibitor is tetramethylammonium in the following concentrations: Curve (1) 0 M; (2) 5.0×10^{-3} M; (3) 9.55×10^{-3} M; (4) 1.37×10^{-2} M; (5) 2.42×10^{-2} M; (6) 5.25×10^{-2} M; (7) 7.88×10^{-2} M; and (8) 1.05×10^{-1} M. In *B* the inhibitor is choline in the following concentrations: Curve (1) 0 M; (2) 1.71×10^{-3} M; (3) 3.27×10^{-3} M; (4) 9.0×10^{-3} M; (5) 1.8×10^{-2} M; (6) 2.7×10^{-2} M; and (7) 3.6×10^{-2} M. As in the other figures, the velocity is in units of 10^{-6} moles min.$^{-1}$/10 ml reaction mixture.

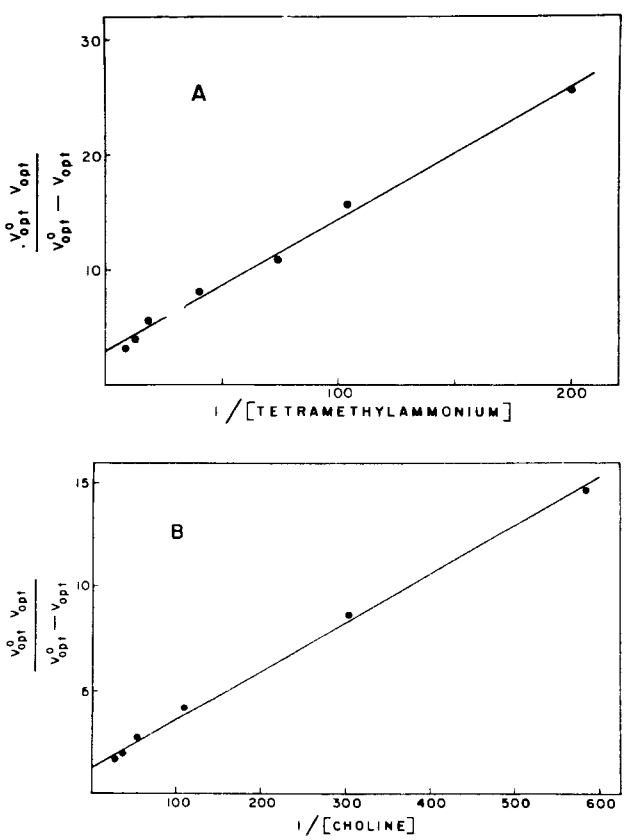

FIG. 3.—A function of the optimum velocity, $v^0_{opt}v_{opt}/(v^0_{opt} - v_{opt})$, in units of 10^{-6} moles min.$^{-1}$, plotted against the reciprocal of the inhibitor concentration (M^{-1}). *A* and *B* show the data for tetramethylammonium and choline respectively.

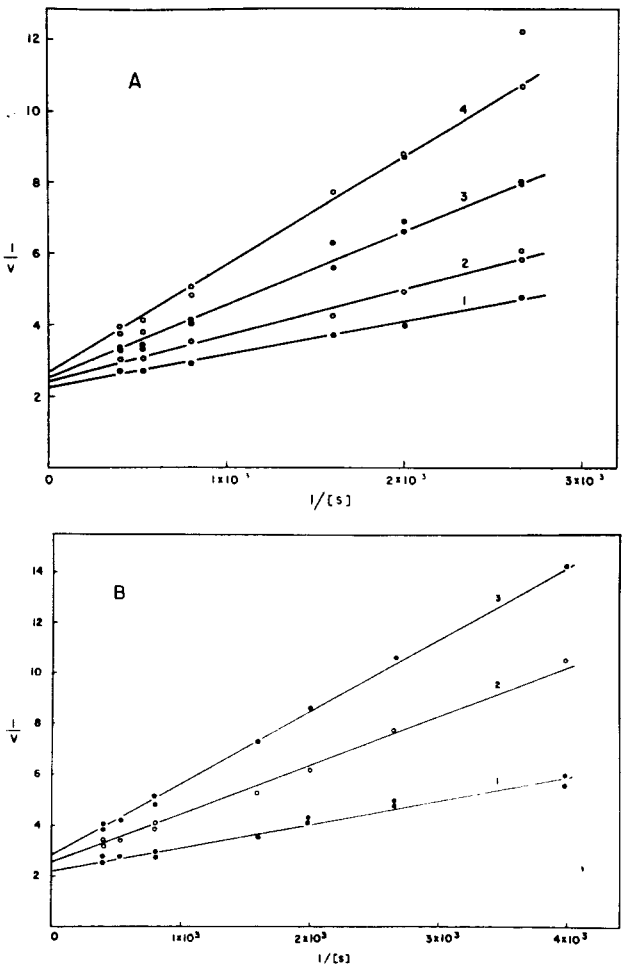

FIG. 4.—Plots of the reciprocal of the reaction velocity against the reciprocal of the substrate concentration (M^{-1}), for inhibition by tetramethylammonium (*A*) and choline (*B*). In *A* the concentrations of tetramethylammonium are: (1) 0 M; (2) 2.13×10^{-3} M; (3) 5.25×10^{-3} M; (4) 1.05×10^{-2} M. In *B* the concentrations of choline are: (1) 0 M; (2) 1.8×10^{-3} M; (3) 3.6×10^{-3} M. The units of $1/v$ are 10^5 min. mole^{-1}.

the $1/v$ intercepts depend to a slight extent on $[I]$, in agreement with equation (10). Information is also theoretically available from the relationship between $[S]_{opt}$ and $[I]$, but, as the precise experimental determination of $[S]_{opt}$ is difficult, especially when $[I]$ is large, it would be unwise to place much reliance on these data.

The experimental results show that both q and $\bar{K}'_i$ have non-zero values, for both the slopes and intercepts of the plots of $v^0_{opt}\,v_{opt}/(v^0_{opt} - v_{opt})$ against $1/[I]$ are positive. The non-zero value for $\bar{K}'_i$ indicates that both b and k'_i/k'_{-i} must have non-zero values. The positive value for q admits of two different interpretations. One is that b is less than unity but greater than zero. The other is that $b = 1$ and that k_{-i} is not much larger than k_3. Since b cannot be determined directly by the methods used here, the relative importance of these interpretations cannot be decided. Both effects may operate to give q a positive value. Main (1961) has shown that k_{-1} is not very much larger than k_2 in systems composed of serum cholinesterase and nitrophenyl esters. This observation lends some plausibility to a suggestion that the desorption of choline or tetramethylammonium from the anionic site may not be much faster than deacetylation.

If q is positive the optimum velocity is lowered by

the inhibitor and a noncompetitive component appears in the inhibition. As we have seen, even if $b = 1$ noncompetitive behavior could result provided that k_{-i} is not much larger than k_3. In general, noncompetition occurs if the inhibitor can form a complex with the enzyme *via* a pathway which is not open to the substrate. In this case an enzyme-inhibitor complex could be formed upon deacetylation of the acetyl enzyme containing a bound inhibitor molecule, whereas no corresponding pathway is open to the substrate, whose complex with the acetyl enzyme is unreactive.

Given that b and k'_i/k'_{-i} are larger than zero and that $\bar{K}'_i$ is smaller than K_i, we should expect the optimum substrate concentration to be approximately related to $[I]$ raised to a power greater than $^1/_2$ but less than unity (see equation 2). It can be shown that the data for choline and tetramethylammonium are consistent with such a relationship.

The Behavior of Acetylcholinesterase Systems in Relation to Particular Inhibitors, Enzymes, Substrates, and Experimental Conditions.—Equation (1) predicts various types of behavior with respect to the optimum substrate concentration and the optimum velocity, depending mainly upon the sizes of q and $\bar{K}'_i$. The effect of the inhibitor on $[S]_{opt}$ does not depend on the size of q, but the effect on v_{opt} does; the larger q is made the lower the optimum velocity becomes in the presence of the inhibitor. If $q = 0$ and $\bar{K}'_i = K_i$, the inhibitor has no effect on the optimum velocity, even though it inhibits strongly at low substrate concentrations.

To determine the predictions of equation (1), let us consider the ratio of the optimum rate in the presence of the inhibitor and the rate at the same substrate concentration in its absence. The optimum velocity is given by equation (8) and the corresponding substrate concentration by equation (2). The velocity at this substrate concentration in the absence of the inhibitor (v_0) is found by substituting (2) into the form of equation (1) in which $[I] = 0$:

$$v_0 = k_2[E]_0 \Big/ \left\{ p + \left(\frac{rKs}{\bar{K}}\right)^{1/2} [(1 + K_i[I])^{1/2}(1 + \bar{K}'_i[I])^{1/2} + 1/(1 + K_i[I])^{1/2}(1 + \bar{K}'_i[I])^{1/2}] \right\} \quad (12)$$

The ratio of equations (8) and (12) is the ratio of the rates in the presence and absence of the inhibitor:

$$\frac{v_{opt}}{v_0} = \frac{p + \left(\frac{rKs}{\bar{K}}\right)^{1/2} \{(1 + K_i[I])^{1/2}(1 + \bar{K}'_i[I])^{1/2} + 1/(1 + K_i[I])^{1/2}(1 + \bar{K}'_i[I])^{1/2}\}}{p + \frac{q\bar{K}'_i[I]}{1 + \bar{K}'_i[I]} + 2(rK_s/\bar{K})^{1/2}\left(\frac{1 + K_i[I]}{1 + \bar{K}'_i[I]}\right)^{1/2}} \quad (13)$$

Whether v_{opt}/v_0 is greater or less than unity depends on the relative magnitudes of the numerator and denominator of (13), and this depends on the relative magnitudes of the following expressions:

$$q(\bar{K}/rK_s)^{1/2}\bar{K}'_i[I] \quad (14)$$

and

$$(\bar{K}'_i[I] - 1)(1 + K_i[I])^{1/2}(1 + \bar{K}'_i[I])^{1/2} + \left(\frac{1 + \bar{K}'_i[I]}{1 + K_i[I]}\right)^{1/2} \quad (15)$$

When (15) is greater than (14) the rate in the presence of the inhibitor (v_{opt}) is higher than that in its absence (v_0), and vice versa. If $K_i > \bar{K}'_i$, (15) becomes negative at very low concentrations of the inhibitor, and is therefore smaller than (14). As $[I]$ increases, (15) becomes larger, its rate of increase at high $[I]$ becoming proportional to the second power of $[I]$. Consequently (15) becomes larger than (14) at sufficiently high $[I]$. We may therefore expect that for positive values of q, and for low values of $[I]$, the optimal rate with the inhibitor will be lower than the rate at the same substrate concentration in the absence of the inhibitor. At high $[I]$ the reverse will be true, the rate being higher in the presence of the inhibitor than in its absence. In practice, however, the concentration of the inhibitor cannot be increased indefinitely, as the kinetic measurements become difficult when the reaction rate is extremely low. For this reason, if q is relatively large, the rate in the presence of the inhibitor may never be observed to exceed that in its absence.

Thus the observed behavior of acetylcholinesterase systems with type II inhibitors should fall into one of three classes, depending on the relative sizes of the constants in equation (1). In one class, for which $q = 0$, $K_i = \bar{K}'_i$, and $b = 1$, the reaction rate rises to the same optimum value regardless of the inhibitor concentration. In a second class of systems, for which q, though larger than zero, is relatively small, the reaction velocity in the presence of the inhibitor rises to a maximum which is higher than that at the same substrate concentration in the absence of the inhibitor, but which is lower than the optimum rate in the absence of the inhibitor. In a third class, for which q is relatively large, the rate is depressed by the inhibitor at all substrate concentrations. These three classes, designated as A, B, and C, are illustrated in Figure 5.

As we have seen, the behavior of a system should depend upon the size of q, as well as $\bar{K}/rK_s$ and $K_i/\bar{K}'_i$. It is apparent that these constants could be affected by the particular inhibitor, enzyme, and substrate, and by the experimental conditions. Experiments have been reported by Augustinsson (1948) which are in agreement with these predictions; examples of each of the three classes, and of the effects of different enzymes, substrates, and inhibitors are listed in Table I. A possible example of the effect of different experimental conditions is the difference in the behavior of systems composed of acetylcholine, choline, and the enzyme from bovine erythrocytes. The behavior with a hemolysate of erythrocytes is of class A, but with a purified enzyme is of class C (see Table I); the hemolysate may affect the medium in which the reaction occurs and so alter the parameters of equation (1).

It is interesting that the behavior with serum, or "pseudo," cholinesterase is very similar to that with the "true" cholinesterase derived from dog brain and bovine erythrocytes.

Nonlinearity of Plots of v_0/v against $[I]$.—If v is the reaction rate in the presence of the inhibitor and v_0 the rate in its absence, it is found that

$$\frac{v_0}{v} = 1 + \frac{K_i[I] + \bar{K}'_i[I]\bar{K}[S](q - rK_s[S])/(1 + \bar{K}'_i[I])}{1 + \bar{K}[S](p + rK_s[S])} \quad (16)$$

This becomes, for very low and very high $[S]$, respectively,

$$v_0/v = 1 + K_i[I] \quad (17)$$

TABLE I

THE BEHAVIOR OF CHOLINESTERASE SYSTEMS AS INFLUENCED BY PARTICULAR ENZYMES, SUBSTRATES, AND INHIBITORS
The three classes of behavior are illustrated in Figure 8. Data from Augustinsson (1948).

Variable	Enzyme Source	Substrate	Inhibitor	Class of Behavior
Enzyme	*Sepia* "liver"	Acetylcholine	Clupeine	A
	Dog brain	Acetylcholine	Clupeine	A
	Bovine erythrocyte (purified)	Acetylcholine	Clupeine	B
	Helix blood	Acetylcholine	Clupeine	B
	Bovine erythrocyte (hemolysate)	Acetylcholine	Choline	A
	Helix blood	Acetylcholine	Choline	B
	Dog brain	Acetylcholine	Choline	C
	Bovine erythrocyte (purified)	Acetylcholine	Choline	C
Substrate	Dog brain	Acetylcholine	Clupeine	A
	Dog brain	Acetyl-β-methylcholine	Clupeine	C
	Horse serum	Acetylsalicylcholine	Clupeine	B
	Horse serum	Benzoylcholine	Clupeine	C
Inhibitor	Dog brain	Acetylcholine	Clupeine	A
	Dog brain	Acetylcholine	Choline	C
	Bovine erythrocyte (purified)	Acetylcholine	Clupeine	B
	Bovine erythrocyte (purified)	Acetylcholine	Choline	C

and

$$v_0/v = 1 + \frac{\bar{K}'_i[I](q - rK_s[S])}{(1 + \bar{K}'_i[I])(p + rK_s[S])} \qquad (18)$$

According to this, plots of v_0/v against $[I]$ should be linear at low $[S]$, while at high $[S]$, v_0/v should approach a maximum as $[I]$ is raised. Such behavior has been observed by Augustinsson (1948) for choline inhibition. At high $[S]$, however, extremely high concentrations of the inhibitor (10^{-1} M) gave rise to a secondary increase in the value of v_0/v. Presumably this secondary increase is due to an additional effect of the inhibitor, possibly not directly related to the active center of the enzyme.

Comparison of Types I and II Inhibitors.—Inhibitors such as tetramethylammonium and choline ions (type II) have been referred to as purely competitive (Krupka and Laidler, 1961), but we now know that there is a small noncompetitive component in their inhibition. Inhibitors such as *cis*-2-dimethylaminocyclohexanol (type I), which show a large noncompetitive component, were referred to as partially competitive and partially noncompetitive. In view of the similarity in the effects of the two types of inhibitors, we should note the reasons for believing that their inhibitory mechanisms are different. One argument depends upon the relationships between v_{opt} and $[I]$. With the type II inhibitor (tetramethylammonium) there is a linear relationship between $v^0_{opt}\, v_{opt}/(v^0_{opt} - v_{opt})$ and $1/[I]$, and, most important, the intercept on the $v^0_{opt}\, v_{opt}/(v^0_{opt} - v_{opt})$ axis is larger than zero. The latter fact was seen to indicate that b is greater than zero. With the type I inhibitor (*cis*-2-dimethylaminocyclohexanol) $1/v_{opt}$ is proportional to $[I]$, as predicted by the model in which EAI does not break down.

An independent argument for the non-zero value of b with type II inhibitors depends upon the experimental observation of class A and B behavior, in which the inhibitor increases the reaction rate at high substrate concentrations. This behavior can be accounted for if the rates at which EAI and EA undergo deacetylation are similar. If EAI does not break down ($b = 0$), the rate expression is as follows:

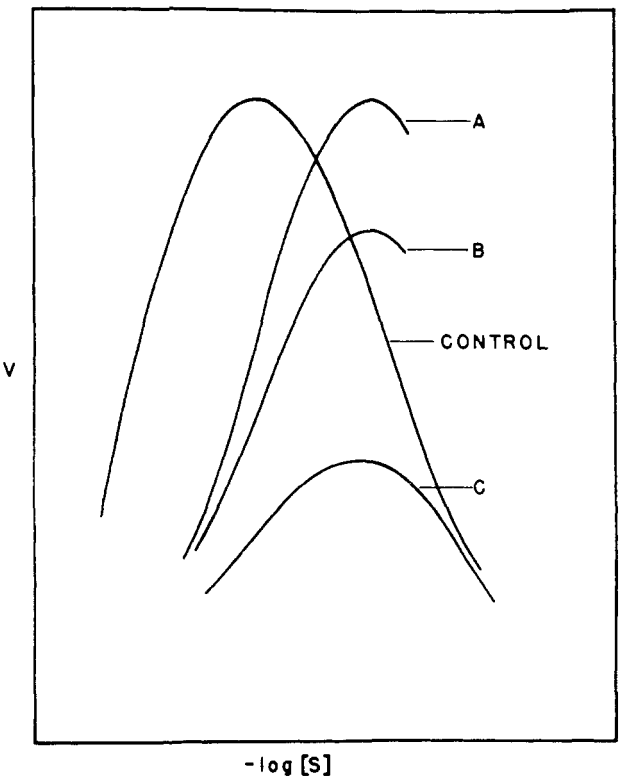

FIG. 5.—The three classes of behavior predicted by the scheme in Figure 1 and observed experimentally with cholinesterase systems by Augustinsson (1948) (see Table I).

This expression cannot account for an increase in the reaction velocity in the presence of the inhibitor, which results only if the term in $K_s[S]$ is divided by a term in $[I]$, that is, if deacetylation is not greatly hindered by a combined inhibitor molecule. Thus, class A and B behavior arise because the inhibitor protects the acetyl enzyme against substrate inhibition. If the substrate inhibits by complexing with the acetyl enzyme, the inhibitor can have this protective action only

$$v = \frac{k_3 E_0 \bar{K}[S]}{1 + K_i[I] + \bar{K}[S]\left\{p + \frac{k_2 k'_{+1} k'_{-c}}{k_3 k'_{-1}(k'_{-c} + ak_3)}[I] + rK_s[S]\right\}} \qquad (19)$$

if it competes with the substrate for the acetyl enzyme without completely blocking deacetylation. To overcome the substrate inhibition entirely, as in class A behavior, the inhibitor must have no effect on the deacetylation rate when bound by the acetyl enzyme.

Conclusion

The postulated mechanism for cholinesterase-catalyzed hydrolysis is in agreement with the experimental relationships between the optimum substrate concentration, the optimum velocity, and the inhibitor concentration. The mechanism explains many previously reported experiments (Augustinsson, 1948) in which diverse types of behavior were observed with different inhibitors, different substrates, and different experimental conditions, and with enzymes from different sources (Fig. 5, Table I). It also explains the small noncompetitive component observed with type II inhibitors such as tetramethylammonium. It is concluded that when the type II inhibitors become bound to the acetyl enzyme they at most partially block deacetylation. In contrast, deacetylation is prevented when the type I inhibitors, such as *cis* - 2 - dimethylaminocyclohexanol, become bound to the acetyl enzyme.

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The α-Chymotrypsin Catalyzed Hydrolysis of a Series of Acylated Glycine Methyl Esters*

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The kinetics of the α-chymotropsin catalyzed hydrolysis of twenty-two acylated glycine methyl esters in aqueous solutions at 25.0°, pH 7.90, and 0.50 M with respect to sodium chloride have been investigated. These data and those for the only previously known substrate of this class, methyl hippurate, have been integrated into a theory developed earlier to explain the structural and stereochemical specificity of α-chymotrypsin for trifunctional substrates of this enzyme.

In 1952 Huang and Niemann reported that the hydrolysis of methyl hippurate was catalyzed by α-chymotrypsin and through inhibition studies provided support for their conclusion that this substrate was hydrolyzed at the same site involved in the hydrolysis of previously recognized substrates of this enzyme. They also proposed that methyl hippurate be regarded as a bifunctional substrate to distinguish it from analogous trifunctional substrates which contained a side-chain as an additional structural feature. This distinction soon received experimental support from the more extended inhibition studies of Huang and Niemann (1953) and from those of Applewhite *et al.* (1958). Applewhite and Niemann (1959), and Hein and Niemann (1962). In these investigations it was found that methyl hippurate could be distinguished from representative trifunctional substrates by the character of the inhibition produced, for example, by indole. With trifunctional substrates the inhibition was fully competitive whereas with the bifunctional substrate it was of the mixed type (Dixon and Webb, 1958). The division of acylated α-amino acid derivatives into the two preceding classes of substrates has recently received further support from the studies of Wallace,[1] who found that whereas systems containing α-chymotrypsin and trifunctional substrates are relatively insensitive to the presence of 9-aminoacridinium ion, those containing a bifunctional substrate are substantially activated.

It is possible to distinguish substrates of any particular class on the basis of differences in the dependence of reactivity upon the structure of the substrate. Although an impressive amount of information of this kind is available for trifunctional substrates (Hein and Niemann, 1961), this is not the case for bifunctional substrates. To remedy this situation we have studied the α-chymotrypsin catalyzed hydrolysis of twenty-two acylated glycine methyl esters. It is the purpose of this communication to describe these experiments, to present further evidence for the distinction between bi- and trifunctional substrates, and to interpret the kinetic behavior of these bifunctional substrates in terms of the theory of the stereo- and structural specificity of α-chymotrypsin developed by Hein and Niemann (1961, 1962) from consideration of the behavior of the analogous trifunctional substrates.

Experimental

The twenty-two substrates were prepared as described below. All melting points were corrected and all analyses were by Dr. A. Elek.

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[1] Wallace, R. A. (1962), unpublished results obtained in these laboratories.