The behavior of hydrolytic enzymes in general and acetylcholinesterase in particular is best understood by assuming that a labile acyl enzyme is formed as an intermediate during hydrolysis (1-3). For acetylcholinesterase the mechanism is:

\[
E + S \rightleftharpoons E\cdot S \rightleftharpoons E\cdot P_1 + P_1
\]

\[
E + I \rightleftharpoons E\cdot I, K_I
\]

\[
E' + I \rightleftharpoons E'\cdot I, K_I'
\]

Here \( E' \) is the acetyl enzyme, \( P_1 \) is choline, \( P_2 \) is acetic acid, \( K_I \) and \( K_I' \) are dissociation constants and the other symbols have their standard meaning.

It is known that reversible inhibitors, for example tetramethylammonium ion, can prevent the reaction of diethylphosphoryl derivatives of acetylcholinesterase with hydroxylamine and other acetylcholinesterase reactivators (7). Since the hydrolysis of the acetyl enzyme is clearly analogous to this reaction, it is reasonable to expect that it should be inhibited by tetramethylammonium ion. Earlier measurements by a rather approximate method (manometric) with a number of simple quaternary ammonium ions did not indicate any displacement of the \( v^{-1} \) intercept (8).

This seeming inconsistency would be explained if \( k_4/k_5 \) were very large. There is evidence, however, although not on a very firm basis, that \( k_4 \) is not very different from \( k_5 \), possibly 1/3 the value of \( k_5 \). Also, if this explanation were true we should lose a highly plausible explanation of substrate inhibition. Another possibility is that these inhibitors are very much more poorly bound by the acetyl enzyme than by the free enzyme and therefore only measurements of high precision have any possibility of revealing the \( v^{-1} \) displacement. There is, in fact, some support for this possibility in that those compounds were found to be bound by the diethylphosphoryl enzyme, on the order of 100 times more poorly than by the free enzyme. This explanation for the failure to detect a \( v^{-1} \) displacement with simple quaternary ammonium ions is reasonable, but we need some positive evidence for the correctness of the general theory and for the theory of substrate inhibition upon which it depends. In this theory of substrate inhibition it is postulated that the hydrolysis of the acetyl enzyme is prevented by the binding of a molecule of acetylcholine (9).

\[
v = \frac{kE0}{1 + K_m/(s) + (s)/K_I'\left(1 + \frac{k_2}{k_4}\right)}
\]
Friess (10) has found that some inhibitors, notably cis-2-di-
methylaminocyclohexanol, show a noncompetitive component; and this compound has been further investigated by Krupka and Laidler (6) in terms of Equation 1. They have shown that the $r^{-1}$ displacement is obtained when acetylcholine is the substrate but not when the substrate is methylaminomethyl acetate. Prior information has indicated that $k_{d}/k_{s}$ was probably rather less than one for acetylcholine and probably rather larger than one for methylaminomethyl acetate (9). This experiment rather strongly suggests the correctness of the theory.

In this paper we report the results of a reinvestigation of the kinetics of inhibition by a precise method. It is shown that the $r^{-1}$ displacement is quite general. We have included compounds which appear to be especially pertinent to the theory of substrate inhibition.

We also report studies on the inhibition of the hydrolysis of methylcarbamyl enzyme by substituted ammonium ions. The methylcarbamyl enzyme has a more convenient half-life (38 minutes) than the carbamyl enzyme (2 minutes). It would appear that methylcarbamyl enzyme would be an analogue of propionyl enzyme and carbamyl enzyme of acetyl enzyme but the distinction is not important because acetyl enzyme and propionyl enzyme appear to have the same kinetic properties, as judged by the fact that the kinetics of the hydrolysis of acetylcholine and propionylcholine are the same (12). Because of the planar structure of the carbamyl function, this group has a smaller effective volume than an acetyl group and this might be reflected in smaller steric effects.

The hydrolysis of methylcarbamyl enzyme and its inhibition are represented by

\[ E^{*} + \text{methylcarbamyl acid} \rightarrow E + \text{hydrolysis of methylcarbamyl enzyme} \]

where the double primes are used to avoid confusion with the acetyl enzyme. The rate of recovery is given in terms of half-times by

\[ t_{l} = t_{0} \left[ 1 + \frac{1}{K_{r}} \right] \]  

**EXPERIMENTAL PROCEDURE**

**Enzyme**—Acetylcholinesterase was prepared from *Electrophorus electricus* by the method of Lawler (13) and had an activity of 0.93 mmole of acetylcholine hydrolyzed per minute (25°, pH 7.0, 0.1 m NaCl, 0.02 m MgCl₂, 0.008% gelatin and acetylcholine concentration $1 \times 10^{-4}$ m) per mg of protein and 3.68 mg of solution. With tetramethylammonium ion, pentyltrimethylammonium ion, and phenyltrimethylammonium ion, the displacement was quite small. In other cases it was much more pronounced and with trimethylammonium ion and dimethylammonium ion, it was prominent. The kinetic constants calculated from Equation 1 are presented in Table I and some typical experimental data are shown in Fig. 1. In those cases where $K_{r}$ is very much smaller (50 to 100 times) than $K_{r}^{*}(1 + (k_{d}/k_{s}))$ only the order of magnitude of the latter can be determined. It is easy to understand that, in cases like tetramethylammonium ion, the displacement was not previously noted.

The dissociation constants for binding with methylcarbamyl enzyme ($K_{r}$) are also given in Table I and some experimental results are shown in Fig. 2. These constants were calculated from Equation 3. In one case, tetramethylammonium ion, the line was distinctly curved. This might be interpreted as indicating that the $E^{*} \cdot I$ complex can react with water to some extent.

Hydrolysis of *Acetylcholine*—The kinetic measurements were made with a Beckman type K automatic titrator modified so that the electrical pulses operate a rotary solenoid coupled to a lead screw which advances the plunger in a 1-ml syringe and thereby delivers aliquots of 0.2 µl of 0.010 m NaOH. Careful “CO₂-free” technique was followed, including a stream of N₂ over the liquid in the reaction vessel.

The reaction was started by the addition of sufficient concentrated acetylcholine solution to make a final concentration of $1 \times 10^{-4}$ m or $2 \times 10^{-4}$ m. After a few minutes of reaction during which only a few per cent of the substrate was hydrolyzed another portion of acetylcholine solution was added to bring the concentration to a new value. In this way 4 or 5 substrate concentrations were used up to a maximal concentration of $1 \times 10^{-3}$ m. Higher concentrations were not used because of substrate inhibition. Small corrections of 2.5% and less were applied for substrate inhibition and the average value of the concentration was used for plotting the data in accordance with Equation 1. The sum of the percentage deviations from the best straight line divided by the number of points minus 2 were generally well less than 1%.

**RESULTS**

A displacement of the $r^{-1}$ intercept was found in all cases. With tetramethylammonium ion, pentyltrimethylammonium ion, and phenyltrimethylammonium ion, the displacement was quite small. In other cases it was much more pronounced and with trimethylammonium ion and dimethylammonium ion, it was prominent. The kinetic constants calculated from Equation 1 are presented in Table I and some typical experimental data are shown in Fig. 1. In those cases where $K_{r}$ is very much smaller (50 to 100 times) than $K_{r}^{*}(1 + (k_{d}/k_{s}))$ only the order of magnitude of the latter can be determined. It is easy to understand that, in cases like tetramethylammonium ion, the displacement was not previously noted.

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ment should be less than that found with dimethylammonium ion alone. It was found, however, that the combined displacement was the sum of the independent displacements. We can there-

\[ K_r K_r' \left( 1 + \frac{k_3}{k_4} \right) \]

\[ K_r (1 + k_4) \]

**Table I**

Dissociation constants for the binding of reversible inhibitors with the free enzyme, \( K_r \), the acetyl enzyme, \( K_r' \), and the methylcarbamyl enzyme, \( K_r'' \)

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Acetylcholine hydrolysis</th>
<th>Methylcarbamyl enzyme hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>([(\text{CH}_3)_2\text{N}]^+ \text{I}^-) (Tetramethylammonium ion)</td>
<td>(1.2 \times 10^{-2})</td>
<td>(~5 \times 10^{-2})</td>
</tr>
<tr>
<td>2</td>
<td>([(\text{CH}_3)_2\text{NH}]^+ \text{Cl}^-) (Trimethylammonium ion)</td>
<td>(4.8 \times 10^{-3})</td>
<td>(4.0 \times 10^{-2})</td>
</tr>
<tr>
<td>3</td>
<td>([(\text{CH}_3)_2\text{NH}]^+ \text{Cl}^-) (Dimethylammonium ion)</td>
<td>(2.6 \times 10^{-2})</td>
<td>(3.2 \times 10^{-2})</td>
</tr>
<tr>
<td>4</td>
<td>([(\text{CH}_3)_2\text{NH}]^+ \text{I}^-) (Pentyltrimethylammonium ion)</td>
<td>(2.0 \times 10^{-4})</td>
<td>(~2 \times 10^{-3})</td>
</tr>
<tr>
<td>5</td>
<td>([(\text{CH}_3)_2\text{N}^- \text{C}_2\text{H}_4\text{COO}]^+ \text{Br}^-) ((\beta)-Trimethylaminopropionic acid ethyl ester)</td>
<td>(2.0 \times 10^{-3})</td>
<td>(2.0 \times 10^{-3})</td>
</tr>
<tr>
<td>6</td>
<td>([(\text{CH}_3)_2\text{N}^- \text{C}_2\text{H}_4\text{COO}]^+ \text{Br}^-) ((\gamma)-Trimethylaminobutyric acid ethyl ester)</td>
<td>(7.7 \times 10^{-4})</td>
<td>(1.1 \times 10^{-2})</td>
</tr>
<tr>
<td>7</td>
<td>([(\text{CH}_3)_2\text{N}^- \text{C}_2\text{H}_4\text{COO}]^+ \text{Br}^-) (Acetylcholine)</td>
<td>(1.4 \times 10^{-4})</td>
<td>(3.2 \times 10^{-2})</td>
</tr>
<tr>
<td>8</td>
<td>(\text{N}^- \text{C}_2\text{H}_4\text{I}^-) (N-Methylpyridinium ion)</td>
<td>(1.1 \times 10^{-4})</td>
<td>(1.2 \times 10^{-3})</td>
</tr>
<tr>
<td>9</td>
<td>(\text{N}^- \text{C}_2\text{H}_4\text{I}^-) (Phenyltrimethylammonium ion)</td>
<td>(5.3 \times 10^{-4})</td>
<td>(~2 \times 10^{-3})</td>
</tr>
</tbody>
</table>

* It appears that \( k_4 < k_3 \) so that this expression approximates \( K_r' \).

† \( K_m \) value, \( K_m = \frac{k_1 + k_2}{k_1} / \left( 1 + \frac{k_3}{k_4} \right) \); see Equation 1.

‡ \( K_r' \left( 1 + \frac{k_4}{k_3} \right) \); see Equation 2.

**Fig. 1.** Lineweaver-Burk plots of the kinetics of the hydrolysis of acetylcholine in the presence of three representative inhibitors and by itself. The displacement of the ordinate intercept indicates a noncompetitive component in the inhibition.

**Fig. 2.** The half-time for the recovery of the methylcarbamyl derivative of acetylcholinesterase by reaction with water in the presence of various concentrations of two reversible inhibitors and acetylcholine. All inhibit the reaction with water.

**DISCUSSION**

It is helpful to consider first the data for the two smallest inhibitors, dimethylammonium ion and trimethylammonium ion. The dissociation constants for binding with the free enzyme are about the same as for binding with the methylcarbamyl enzyme and are also about the same as the combination \( K_r' \left( 1 + \frac{k_4}{k_3} \right) \). There is certainly no reason to suppose that binding to the acetyl enzyme would be stronger. We may therefore conclude that \( k_4 \) is rather smaller than \( k_3 \) and that \( K_r' \left( 1 + \frac{k_4}{k_3} \right) \) approximates \( K_r' \). Then we have the reassuring result that
binding with the enzyme and with its two derivatives has about the same strength. Previous observations on the temperature dependence of the rate of enzymic hydrolysis of acetylcholine had also suggested that $k_4$ was smaller than $k_3$, possibly $\frac{1}{3}$ the value (9).

It is interesting that the affinity of tetramethylammonium ion for the free enzyme is about four times greater than that of trimethylammonium ion. In earlier work, the $I_30$ values when ($S$) = $4 \times 10^{-3}$ M were found to be about the same. The present measurements of the dissociation constants clear up a minor discrepancy which was recognized after the earlier $I_30$ values were published. It had been concluded that the fourth methyl group in tetramethylammonium ion projected into the solution and was without binding properties. This physical picture is what one would, a priori, assume and hardly needed much confirmation. Upon reflection, however, it is clear that the nonbinding methyl group can be selected in four ways and that tetramethylammonium ion ought, therefore, to be about four times better bound than trimethylammonium ion. (A more detailed analysis does not seem justified.)

We now consider the theory of substrate inhibition. From the fact that the hydrolysis of the carbamyl enzyme can be inhibited by reversible inhibitors, it is clear that the hydrolysis of the acetyl enzyme should also be inhibited. This is substantiated by the $v^{-1}$ displacement as already discussed. The only questions remaining are, “can acetylcholine inhibit the hydrolysis of the acetyl enzyme, and if so, can it do so at concentrations where substrate inhibition occurs?” Studies with the carbamyl enzyme showed that acetylcholine could prevent its hydrolysis and yielded a dissociation constant of $2 \times 10^{-2}$ M. This is very nearly the same as the dissociation constant obtained for binding with the acetyl enzyme when that constant is evaluated in terms of Equation 2. Moreover, it is right in line with the dissociation constants for the binding of the acetyl enzyme with the reverse esters, compounds No. 5 and 6 (Table I), which bear an obvious relationship to acetylcholine but are not substrates. These results leave little doubt that the correct explanation of substrate inhibition is that the deacylation of the acetyl enzyme is prevented by the binding of a molecule of acetylcholine.

**SUMMARY**

Kinetic measurements of relatively high precision show that reversible inhibitors of acetylcholinesterase have a noncompetitive component. These findings are consistent with deductions arising from the intermediate formation of an acetyl enzyme in enzymic hydrolysis, for it follows that it should be possible to inhibit the deacylation and this introduces the noncompetitive component. In support of this theory, reversible inhibitors are shown to inhibit the hydrolysis of methylcarbamyl enzymes, a reaction analogous to the hydrolysis of the acetyl enzyme. It is shown that acetylcholine inhibits the hydrolysis of the methylcarbamyl enzyme and, therefore, would be expected to inhibit the hydrolysis of the acetyl enzyme. This would produce substrate inhibition. The binding constant of acetylcholine with the acetyl enzyme is nearly the same as with the carbamyl enzyme.

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