ACETYLCHOLINESTERASE

X. MECHANISM OF THE CATALYSIS OF ACYLATION REACTIONS*

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Ester synthesis catalyzed by esterases has been the subject of many investigations (1, 2). The reversal of acetylcholine hydrolysis by serum esterase has been described by Abderhalden and Paffrath (3) and Ammon and Kwiatkowski (4). The availability of a highly active and purified acetylcholinesterase and the development of a chemical method for the determination of acetylcholine in the presence of excess of acetate and choline made possible a quantitative analysis of the equilibrium (5). In a preceding paper it has been described that this equilibrium shifts markedly in favor of esterification of choline and acetate with decreasing pH (6). Acethydroxamic acid formation was also found to be catalyzed by the enzyme. The optimum for this reaction was found to be pH 6.3.

The picture of the active surface of acetylcholinesterase developed in a series of papers (7-10) suggests that the enzyme contains a basic group capable of reacting with the electrophilic carbon of the carbonyl group of acetylcholine. This reaction occurs similarly with other compounds which are either substrates or inhibitors. The stronger the electrophilic character of the carbon, the greater the reaction with the enzyme. This was observed with a series of derivatives of nicotinic acid in which the electrophilic character of the carbonyl carbon was progressively increased while other essential structural features remained unaltered. The experiments suggest that for the catalysis of formation of choline esters and of hydroxamic acid by acetylcholinesterase an ester should be a more favorable substrate than the corresponding carboxylate ion. This hypothesis implies that during the hydrolysis of the ester the same, or a similar, intermediary is formed as that which occurs between acid and enzyme. Experiments will be described which test this hypothesis.

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Methods

Preparation of Enzyme—440 gm. of electric organ of Gymnotorpedo occidentalis (Storer), which had been stored under toluene for 3 years, were homogenized in a Waring blender with 5 per cent ammonium sulfate solution and repeatedly extracted until the total volume of extract was 2660 ml. Sufficient ammonium sulfate was added to raise the concentration to 14 per cent. The precipitate which formed overnight was discarded. Protein was precipitated from the supernatant solution by adding ammonium sulfate until the concentration reached 36 per cent. The precipitate so obtained was redissolved by adding 180 ml. of H₂O. Sufficient ammonium sulfate was added to raise the concentration to 15 per cent; the mixture was centrifuged and the precipitate discarded. Ammonium sulfate was added to the supernatant until the concentration reached 30 per cent. The mixture was centrifuged, the supernatant discarded, and 36 ml. of water were added to the precipitate. The 40 ml. of solution so obtained were dialyzed against 0.025 M phosphate buffer. The resulting enzyme solution hydrolyzed about 4.6 gm. of acetylcholine per ml. per hour and contained about 1.8 mg. of protein per ml.

Determination of Hydroxamic Acid and Choline Esters—The hydroxamic acid formed was determined colorimetrically by the addition of HCl and ferric chloride as described previously (11). The chemical determination of the choline ester formed from monobutyryl and ethyl acetate is difficult, since the amount of the choline ester is small compared with the relatively large amount of the starting esters. Therefore, the frog rectus abdominis test for acetyl- and butyrylcholine has been used.

Results

Hydroxamic Acid Formation—Table I summarizes a few typical experiments in which hydroxamic acid formation from acids and esters at different pH levels was studied. The figures show that the formation from esters is much faster than from acids. The pH optimum for the acids was found to be consistent with the value 6.3 reported previously. The rate of hydroxamic acid formation in one experiment at pH 7.0 and in several experiments at pH 7.5 was low. These figures are not included in Table I. The data presented in Table I are the results of one experiment, whereas the other results were obtained with a slightly different enzyme preparation. The rate of formation at pH 5.5 was higher than that reported in the preceding paper (6). Propionylhydroxamic acid formation showed a similar pH dependence. In contrast to the acids, the hydroxamic acid formation from esters increased rapidly with increasing pH. At 8.0 or higher, hydroxamic acid formation is difficult to measure, since at this pH level
non-enzymatic hydroxamic acid formation is very rapid. At pH 7.5, non-enzymatic hydroxamic acid formation was about 10 per cent of the enzyme-catalyzed synthesis. In evaluating the results, it may be noted that the concentrations of the various esters were not identical because of their limited solubility in water and were markedly lower than the concentrations of the acids.

**Table I**

*Hydroxamic Acid Formation from Acids and Esters Catalyzed by Acetylcholinesterase at Various pH Levels*

The incubation mixture contained, per ml., 0.25 ml. of enzyme, prepared from electric tissue of torpedo. The enzyme solution contained about 1.8 mg. of protein per ml. and hydrolyzed about 4.6 gm. of acetylcholine per ml. per hour. A control was always run without enzyme. The hydroxylamine concentration was 0.6 M. Phosphate buffer was used for adjusting the pH. At the end of the incubation period, HCl and ferric chloride were added for the calorimetric determination. In the second experiment with ethyl acetate, the enzyme was slightly more diluted than in the others.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>Incubation</th>
<th>pH 5.5</th>
<th>pH 6.5</th>
<th>pH 7.5</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium acetate</td>
<td>0.5 M</td>
<td>60 min.</td>
<td>1.54 μM</td>
<td>2.47 μM</td>
<td>0.4 μM</td>
<td></td>
</tr>
<tr>
<td>&quot; propionate</td>
<td>0.5 M</td>
<td>120 min.</td>
<td>2.62 μM</td>
<td>4.10 μM</td>
<td>0.0 μM</td>
<td>0.4 μM</td>
</tr>
<tr>
<td>&quot; butyrate</td>
<td>0.5 M</td>
<td>60 min.</td>
<td>0.66 μM</td>
<td>0.77 μM</td>
<td>0.4 μM</td>
<td>0.1 μM</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.17 M</td>
<td>15 min.</td>
<td>10.4 μM</td>
<td>16.0 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; propionate</td>
<td>3 μM</td>
<td>30 min.</td>
<td>14.5 μM</td>
<td>21.4 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; butyrate</td>
<td>6 μM</td>
<td>60 min.</td>
<td>17.5 μM</td>
<td>25.3 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; propionate</td>
<td>0.05 M</td>
<td>3 min.</td>
<td>0.3 μM</td>
<td>1.4 μM</td>
<td>3.0 μM</td>
<td>6.2 μM</td>
</tr>
<tr>
<td>&quot; butyrate</td>
<td>0.05 M</td>
<td>60 min.</td>
<td>2.02 μM</td>
<td>2.63 μM</td>
<td>3.66 μM</td>
<td></td>
</tr>
<tr>
<td>&quot; propionate</td>
<td>120 min.</td>
<td>2.91 μM</td>
<td>2.26 μM</td>
<td>3.66 μM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The rate of formation is roughly proportional to the substrate concentration, as may be seen from the figures in Table II. When the differences in concentration are taken into account, the rate of hydroxamic acid formation is of the order of 100 to 150 times as rapid with an ester as with the corresponding acid at pH 6.5, which is the optimum for the acid but not for the ester. Since the ester reacts at pH 7.5 about twice as rapidly, the difference of the maximum rate is still greater.

The reaction velocity decreases rapidly with time when esters are the substrates, as is seen in Table I. This must be attributed to the rapid
enzymatic hydrolysis of the ester which proceeds simultaneously with the slower hydroxamic acid formation. Therefore, for quantitative evaluation relatively short reaction times must be selected. For the short periods of reaction used in the experiments of Table II the decrease in velocity at the second reading is apparent but not marked. For periods of 3 to 6 minutes (see Table I) the rate was constant. The decrease in reaction velocity is much less when sodium acetate is the substrate.

Monobutyrin also reacts quite readily though less rapidly than ethyl acetate or ethyl propionate, but the non-enzymatic reaction is also quite high. Thus, whereas the non-enzymatic hydrolysis of the butyryl ester is much higher than that of the acetyl or the propionyl ester, the enzymatic synthesis decreases rapidly in the order acetate > propionate > butyrate; i.e., in the same order as is observed in the hydrolysis of choline esters. Acetamide, n-butyramide, and nicotinamide were also tested. No enzymatic reaction was observed under the experimental conditions. Non-enzymatically, however, the amides react at pH 6.5 with hydroxylamine. This reaction will be analyzed in a separate paper.

**Table II**

Hydroxamic Acid Formation As Function of Substrate Concentration

The reaction times were varied in order to get the lowest but still accurate value of hydroxamic acid formed in the incubation mixture. The enzyme was diluted four times for the reaction with ethyl acetate; therefore, the values obtained have been multiplied by 4. 0.025 M phosphate buffer at 6.5 was used. The ionic strength was kept constant by compensating with NaCl when required. The hydroxylamine concentration was 0.6 M; $T = 23^\circ$.

<table>
<thead>
<tr>
<th>Ethyl acetate</th>
<th>Sodium acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>Reaction time</td>
</tr>
<tr>
<td>$\mu M$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$M$</td>
<td>min.</td>
</tr>
<tr>
<td>0.17</td>
<td>6</td>
</tr>
<tr>
<td>0.13</td>
<td>12</td>
</tr>
<tr>
<td>0.085</td>
<td>9</td>
</tr>
<tr>
<td>0.043</td>
<td>20</td>
</tr>
<tr>
<td>0.043</td>
<td>30</td>
</tr>
</tbody>
</table>

Transesterification—From the above results it became evident that in any synthesis catalyzed by acetylcholinesterase the ester should be superior to the corresponding acid. When this principle is applied to the esterifica-
tion of choline with acetate, it may be expected that the enzyme will catalyze transesterification reactions of the type

$$\text{RCOOR'} + \text{R''OH} \rightleftharpoons \text{RCOOR''} + \text{R'OHN}$$

However, such a synthesis is complicated by the fact that the enzyme will simultaneously split both the substrate ester and the product ester. A steady state will be reached in which the rate of formation equals that of hydrolysis of the new ester. The concentration at the steady state may persist for some time, but will eventually decline, due to the decrease in concentration of starting ester.

Fig. 1 represents the results of two experiments in which monobutyrin and choline were incubated with the enzyme. The steady state seems to be reached in about 3 to 4 hours. The usual controls, run simultaneously, included one containing sodium butyrate instead of the ester. No butyrylcholine formation was measurable under the experimental conditions in any of the controls.

It has been previously described that one of the characteristic features of acetylcholinesterase which distinguishes this enzyme from other esterases...
is the decrease of the rate of hydrolysis with increasing length of the acyl chain (12, 13). In the case of torpedo enzyme, propionylcholine was described as being hydrolyzed under the experimental conditions at a rate about one-third of that of acetylcholine. Butyrylcholine was not measurably hydrolyzed under the same conditions (14). This, of course, does not indicate absolute inability of the enzyme to attack butyrylcholine. It would be surprising if the enzyme were completely unable to attack this ester. We have studied this question and have found that the concentration of enzyme must be 140 times as high as for acetylcholine in order to split butyrylcholine at the same rate.

In an experiment in which ethyl acetate and choline were used as substrates the steady state was reached much faster than with monobutyrin, in about 10 minutes, even though only one-twelfth of the enzyme concentration was used. The maximum value, however, was lower; 5.5 γ of acetylcholine were formed per ml. of incubation mixture in 5 minutes and 7 to 8 γ in 10, 20, and 40 minutes. After 80 minutes the concentration had declined to 4.5 γ. However, in this case the concentrations of choline and ester were much lower, 0.03 and 0.28 M respectively. The steady state is determined by the rate of formation which depends upon the concentrations of the enzyme and the two substrates, and by the rate of hydrolysis of ester formed which depends upon the concentration of the enzyme and this ester. The concentration of enzyme is of course the same for the two opposing reactions and will therefore not determine the level of the steady state. The concentration of ethyl acetate and choline was much lower than in the experiments presented in Fig. 1. Therefore, the steady state was reached at a lower level, but the difference of the two levels was of the order of magnitude which would be expected from the difference of the substrate concentrations.

**DISCUSSION**

As anticipated, the rate of formation of enzyme-catalyzed hydroxamic acid is some 100 times more rapid with esters than with the corresponding carboxylate ions. While the carbonyl carbon of carboxylate ion has little electrophilic character, the carbon of undissociated acid is markedly electrophilic. In accordance with the mechanism proposed (9), it would be expected that the actual reactant is the undissociated carboxylic acid and that the rate should be about the same as with the ester. At pH 6.5 there is only about 1 per cent undissociated acid. Since the rate is approximately proportional to the concentration of the reactant, ester and undissociated acid are about equally effective.

Apparently during the enzymatic hydrolysis of an ester an intermediary is formed which may react with either water (or its ions) or with hy-
droxylamine or choline. Any reaction with $\text{H}^+$ or $\text{OH}^-$ is not rate-controlling. Reaction with water results in hydrolysis, while reaction with hydroxylamine produces hydroxamic acid, and with choline it produces choline esters. The relative amounts of the different products depend upon the relative concentrations of these reactants.

The mechanism of enzyme interaction may be outlined as follows:

$$\text{II} - \text{G} + \text{R} - \text{O} - \text{OR}' \rightleftharpoons \text{R}' - \text{O} - \text{C} - \text{O}(-)$$

where $\text{G}$ is that part of the active enzyme surface which contains a basic group, represented by the pair of unshared electrons, and an acid group, represented by the hydrogen atom. The enzyme-substrate complex as shown here is the fundamental structure. The actual structure may be one of a number of more probable minor variations, containing protons or hydrogen bonds joined to one or both oxygens. *A priori* there appear to be several similar mechanisms for the reaction of the complex with nucleophilic reagents. One possible mechanism is the internal elimination of R'OH.

$$\text{II} - \text{G} \rightleftharpoons \text{G}$$

$$\text{R} - \text{O} - \text{C} - \text{O}(-) \rightleftharpoons \text{R} - \text{C} - \text{O}(-) + \text{R'}\text{OH}$$

The same intermediate (A) can be formed from the acid molecule as from the ester. This intermediate can react with water (as shown) or with hydroxyl ion, hydroxylamine, or choline (or with other similar nucleophilic reagents). The formation of the intermediate is the rate-controlling step.

The dependence of the velocity upon pH and reactant concentration can
be developed mathematically if we take into account the acidic dissociation of all reactants including the enzyme. The method of the stationary state along with the experimental observation that the velocity is approximately proportional to the concentration of the reactants yields

\[ v = \frac{K(S)(B)}{H^+ + 1 + \frac{K_{EH^+}}{H^+}} \]

where \( v \) is velocity, \( S \) may be either acid or ester, and \( B \) may be \( \Pi_3O^- \), \( OH^- \), \( H_2NOH \), or choline. \( K_{EH^+} \) and \( K_{EB^+} \) are dissociation constants for the enzyme. \( (S) \) is the concentration of the actual reactant and therefore the stoichiometric concentration \( (S)^0 \) of an ester but the undissociated acid concentration for the salt of a carboxylic acid. \( (B) \) is the stoichiometric concentration \( (B)^0 \) for choline but the base concentration for hydroxylamine. Thus

\[ (S) = (S)^0 \frac{H^+}{K_a + (H^+)} \]

\[ (B) = (B)^0 \frac{K_{B^+}}{K_{B^+} + (H^+)} \]

where \( K_a \) is the acid dissociation constant for the fatty acid and \( K_{B^+} \) is the acid dissociation constant of \( (NH_3OH)^+ \). For carboxylic acid \( K_a \cong 2 \times 10^{-5} \), for hydroxylamine \( K_{B^+} \cong 8 \times 10^{-7} \) and \( K_{EH^+} \cong 7 \times 10^{-7} \), \( K_{EH} \cong 5 \times 10^{-10} \). For the reaction of acid plus hydroxylamine the calculated velocity is a maximum at about pH 6.5, as in fact is found experimentally.

For ester and hydroxylamine the calculated velocity is a maximum at about pH 8.5. The pH behavior should therefore be distinctly different from reactions with the salt. The velocity was found to increase with higher pH up to 7.5, which is, as already mentioned, the highest pH at which the enzymatic velocity could be evaluated.

From the reaction mechanism postulated above, it was possible to conclude that the esterase would catalyze hydroxamic acid formation with esters and at a rate far exceeding that of the reaction with corresponding salts. The very different pH behavior with esters and salts is also explained. It was also expected that esters would react enzymatically with choline to produce choline esters. On the basis of this mechanism it would be anticipated that acetylcholinesterase is capable of catalyzing oxygen exchange between water and carboxylic acids. This possibility will be investigated experimentally.

In the hydrolysis of choline esters catalyzed by torpedo acetylcholin-
esterase, the acetate is hydrolyzed about 3 times as fast as the propionate and about 140 times as fast as the butyrate. These relative velocities are maintained in the reactions of the acids and esters with hydroxylamine and choline, indicating that the rate-controlling steps are the same or similar in all these reactions.

SUMMARY

Several acylation reactions catalyzed by purified acetylcholinesterase have been investigated. The picture of the active enzyme surface recently developed has been used for an analysis of the underlying mechanism.

1. Formation of hydroxamic acid from ethyl acetate is catalyzed by the enzyme some 100 times as fast as from acetic acid.

2. The rates are in both cases approximately proportional to the concentration of the reactants.

3. Hydroxamic acid formation from esters, containing 3- and 4-carbon atoms in the acyl chain, is catalyzed at a much lower rate than those containing 2-carbon atoms. The ratio of the rates is approximately the same as that observed for choline ester hydrolysis.

4. Whereas there is an optimum at pH 6.5 for hydroxamic acid formation from carboxylic acids, the reaction velocity with esters increases with pH.

5. The experimentally found pH optimum for carboxylic acid agrees with that calculated on the basis that the undissociated species is the actual reactant. This is consistent with the assumption that the carbon of the carbonyl must be markedly electrophilic to react with the basic group in the active enzyme surface.

6. In the presence of choline, the enzyme is capable of transferring the acyl group from the ester to the alcohol. Hydrolysis proceeds simultaneously.

7. In the discussion of the mechanism it is assumed that an intermediary is formed which may react either with water (or its ions) or with hydroxylamine or choline. The substance reacting with this intermediary must be nucleophilic.

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