Catalysis by acetylcholinesterase: Evidence that the rate-limiting step for acylation with certain substrates precedes general acid-base catalysis

(enzyme mechanism/diffusion control/induced-fit conformational change/pH dependence/deuterium oxide isotope effects)

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Communicated by David Nachmansohn, June 9, 1975

ABSTRACT Inferences about the catalytic mechanism of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) are frequently made on the basis of a presumed analogy with chymotrypsin, EC 3.4.21.1. Although both enzymes are serine hydrolases, several differences in the steady-state kinetic properties of the two have been observed. In this report particular attention is focused on the second-order reaction constant, $k_{cat}/K_{app}$. While the reported pH dependence and deuterium oxide isotope effect associated with this parameter for chymotrypsin is generally consistent with simple models involving rate-limiting, general acid-base catalysis, this study finds a more complicated situation with acetylcholinesterase. The apparent $pK_A$ of $k_{cat}/K_{app}$ for acetylcholinesterase varies between 5.5 and 6.3 for neutral substrates and involves nonlinear inhibition by $[H^+]$. Deuterium oxide isotope effects for $k_{cat}/K_{app}$ range from 1.1 for acetylcholine to 1.9 for phenylacetate. The bimolecular reaction rate appears rate-limiting for acetylcholine at low concentrations, while a rate-limiting induced-fit step is proposed to account for apparent $pK_A$ values and low deuterium oxide isotope effects associated with low concentrations of phenyl acetate and isoamyl acetate.

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is classified as a serine hydrolase along with other esterases and peptidases which show essentially irreversible active-site phosphorylation (see ref. 1). Equivalent-weight determinations and peptide analysis indicate that phosphorylation occurs only at a single serine residue, and the amino-acid sequence about this residue shows significant homology among the enzymes in this class (1, 2). Three-dimensional structures of several serine hydrolases have been determined by x-ray crystallography, and further striking structural similarities have thus been revealed (3). The native polypeptide conformations of chymotrypsin (EC 3.4.21.1), trypsin, and elastase are nearly superimposable. Blow et al. (4) have reported that a dominant feature of these structures is a "charge-relay" system of hydrogen bonds formed by the active site serine hydroxyl, a histidine imidazole side chain, and a carboxylate side chain in linear array. The evolutionary importance of this charge relay is demonstrated by its identification in subtilisin and other serine hydrolases structurally unrelated to chymotrypsin (see ref. 3).

The discovery of the charge-relay structure coincides with previous inferences about the catalytic mechanism of serine hydrolases from kinetic studies. A minimal catalytic mechanism involves the enzyme species in Scheme 1.

The proposed intermediates include the initial Michaelis complex E-RX and the acyl enzyme ER, for which evidence has long been obtained (5, 6, 1). The pH dependence of substrate hydrolysis for chymotrypsin and other serine hydrolases suggests general acid-base catalysis by a group in the free enzyme with a $pK_A$ of 6 to 7. Furthermore, Hammett relationships with positive rho values are found with chymotrypsin both for deacetylation (7) and acylation (8) reactions and indicate rate-limiting general base catalysis. Decylation rates are typically reduced in deuterium oxide by factors of 2 to 3 (9), in agreement with this indication. Partial protonation generally accompanies loss of the leaving group during acylation (10). Crystallographic analysis of acyl chymotrypsins has suggested that His-57 acts successively during acylation, first as a general base for the attack of Ser-185 on the carbonyl carbon and then as a general acid to assist loss of the leaving group (11). Because of the symmetry of the proposed action of His-57, deacylation is presumed to occur by a similar process.

In the absence of the three-dimensional structure for acetylcholinesterase, inferences about its mechanism have been based both on studies of its substrate catalysis and on analogies drawn from chymotrypsin (see refs. 1, 12, and 13). Yet aside from the similarities which classify it as a serine hydrolase, acetylcholinesterase differs significantly from chymotrypsin both in size and in catalytic properties. 11S acetylcholinesterase is a tetramer of essentially identical catalytic subunits, each with a molecular weight of about 75,000 (14). The active site includes an anionic group which aids in the binding of cationic substrates (see refs. 1, 12, and 13). It is also an esterase as opposed to a peptidase; acetylaceetylcholinesterase is hydrolyzed some 10^6 times faster than acetylchymotrypsin (ref. 15, and see Table 1). Although chymotrypsin is rapidly acylated by specific ester substrates (e.g., ref. 16), its catalytic machinery appears to have evolved for the particular stabilization of the transition state for acylation with specific amides, especially those with amino-acid amide leaving groups (17).

To decide whether the mechanism in Scheme 1 can adequately account for observations on acetylcholinesterase, some properties of the experimental parameters derived from Scheme 1 have been examined in this paper. Under steady-state conditions of substrate hydrolysis ([RX] > $E_{tot}$, where $E_{tot}$ is the enzyme normality), the kinetic parameters $k_{cat}$ and $K_{app}$ are defined by Eq. 1, where $v$ is velocity.

$$ v = \frac{k_{cat}E_{tot}}{1 + K_{app}/[RX]} $$

[1]

The pH dependence of $k_{cat}/K_{app}$ (the second-order rate) and $k_{cat}$ (the first-order rate) have long been formulated for
Table 1. Apparent pKₐ values associated with acetic acid ester substrates of acetylcholinesterase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pKᵦ₁</th>
<th>pKᵦ₂</th>
<th>pKᵦ₁&quot;</th>
<th>pKᵦ₂&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>6.3</td>
<td>&gt;10.5</td>
<td>6.5</td>
<td>&gt;10.5</td>
</tr>
<tr>
<td>0.1 M NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyl acetate</td>
<td>5.5</td>
<td>10.5</td>
<td>6.56</td>
<td>&gt;10.5</td>
</tr>
<tr>
<td>0.1 M NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>5.5</td>
<td>9.8</td>
<td>6.1</td>
<td>10.1</td>
</tr>
</tbody>
</table>

Values were determined as outlined in Materials and Methods.

Scheme 1 under the assumption that all reversibly linked species are equilibrated (18). If protonation of a single group inhibits enzyme acylation, as indicated in Scheme 1, the pH dependence of kₐcat/Kₐapp gives the pKᵦ₁ of this group in the free enzyme; the apparent pKᵦ₂ of kₐcat is a weighted average of the pKᵦ₁ and pKᵦᵡ values for this group in E-RX and ER (see ref. 12). The pH dependence of chymotrypsin is quite consistent with this formulation. A pKᵦ₁ of 6.8 for kₐcat/Kₐapp is observed for virtually all chymotrypsin substrates (9, 19); a similar pKᵦᵡ is obtained for most E-RX species and a pKᵦᵡ of 7.0-7.2 is seen with acetylchymotrypsin ER (9). In contrast, apparent pKᵦ₁ values which vary between 5.5 and 6.3 have been reported by Krupka (20, 21) for partially purified bovine erythrocyte acetylcholinesterase. A pKᵦᵡ of 5.2-5.5 for E-RX was inferred from all substrates for which acetylation is rate-determining, while deacetylation-limited substrates indicated a pKᵦᵡ of 6.3 for the acetyl enzyme ER. Of particular interest in these acetylcholinesterase studies is the suggestion that protonation of two groups in the free enzyme can affect activity (21).

In this report a variability in the pKᵦ₁ of kₐcat/Kₐapp for several substrates of highly purified eel 11S acetylcholinesterase is confirmed. These pKᵦ₁ values and observed deuterium oxide effects provide new information about the acylation of acetylcholinesterase.

MATERIALS AND METHODS

Acetylcholinesterase from electric organs of the eel Electrophorus electricus was purified as an 11S species free of detectable protein contaminants as described previously (14). Acetic acid ester substrates were commercially available reagent grades; phenyl acetate and isoamyl acetate were redistilled and p-nitrophenyl acetate was recrystallized before use. p-Nitrophenyldiethylphosphate (Paraoxon) and deuterium oxide were from Sigma. Steady-state kinetic investigations at 25°C and computer analyses of the resultant 1/ν versus 1/[RX] plots were carried out essentially as described previously (22). The reciprocal of the slope of such plots gives the relative kₐcat/Kₐapp, and the reciprocal of the intercept, the relative kₐcat. Reaction volumes with the pH stat were either 5 or 10 ml, and standardized 0.005-0.10 N NaOH was the titrant. The solvent was 0.1 M NaCl unless otherwise noted, and the pH was adjusted with standard buffers. Spectrophotometric assays were also used with phenyl acetate at pH 8.0 (0.1 M sodium phosphate, Δₑ₂₇₀ 1400) and with p-nitrophenyl acetate both at pH 8.5 (0.1 M NaCl, 0.05 M Tris-HCl, Δₑ₄₀₀ 16,000) and at pH 5.0-8.0 at the p-nitrophenol-p-nitrophenoxide isosbestic point (0.1 M NaCl, 0.05 M sodium phosphate, or 0.05 M sodium acetate, Δₑ₅₄₇ 5000). Rates were corrected for the nonstoichiometric release of protons for acetate at low pH and for phenol and p-nitrophenol at high pH, both in H₂O and D₂O.

The second order phosphorylation rate for p-nitrophenyl-diethylphosphosphate was determined by monitoring the simultaneous hydrolysis of either methyl acetate or p-nitrophenyl acetate. Conditions were defined such that the ratio of the velocity of hydrolysis of the acetic acid ester (at a concentration far below its Kₐapp) to the free enzyme normality was effectively constant during the phosphorylation reaction; this permitted direct continuous determination of the phosphorylation rate by a technique previously applied to carboxymoylating agents (23, 22; see ref. 13).

The effects of pH or high ionic strength on the kinetic parameters kₐcat/Kₐapp and kₐcat were assessed in terms of R. For pH studies, R is defined as the ratio of the value of the kinetic parameter at a given pH to that at pH 8.5. For ionic strength variations, R is the ratio at a given ionic strength to that at 0.1 M NaCl. Values of the apparent inhibition constants Kᵦ₁ and Kᵦᵡ were estimated visually from the initial slopes of plots of 1/R versus [H+] (22). Curvature in these plots precluded the computer analysis used previously (22).

RESULTS

General Effect of pH on the Hydrolysis of Acetic Acid Esters. The pH dependence of phenyl acetate hydrolysis is shown in Fig. 1. Values of pKᵦ and pKᵦᵡ estimated from these data are shown in Table 1. A clear distinction of approximately one pK unit between pKᵦ₁ and pKᵦᵡ for phenyl acetate in 0.1 M NaCl is observed. Values of pKᵦ₁ and pKᵦᵡ for acetate are also shown in Table 1, and a much smaller difference is observed. The agreement of pKᵦᵡ for both substrates is expected, because kₐcat for both is thought to reflect the deacylation rate k₀ (see refs. 1, 12, and note similarity of kₐcat in Table 2). The difference in pKᵦ₁ between phenyl acetate and acetylcholine is consistent with the observations of Krupka on the erythrocyte enzyme (20); slight differences in assay solvent prevent a precise quantitative comparison of the two enzymes.

The requirement of an acidic group with a pKᵦ of 9-10.5 for enzyme activity has been noted by several investigators (see ref. 1). The data in Table 1 with 0.1 M NaCl indicate that such a group has higher pKᵦ₂ and pKᵦᵡ values than some previous estimates. No decrease in the apparent kₐcat/Kₐapp for acetylcholine is observed up to pH 10.25, in contrast to a previous report (24). Only when assays are conducted in 1.0 M NaCl do the pKᵦ₂ and pKᵦᵡ values for this group become clearly discernible.

In 1.0 M NaCl the apparent values of pKᵦ₁", pKᵦ₂, and pKᵦᵡ" are reduced by some 0.5 pK units. A similar observa-
Table 2. Deuterium oxide effects on \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_{\text{app}} \) and pK\(_{a1}\) of \( k_{\text{cat}}/K_{\text{app}} \) for several substrates of acetylcholinesterase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>rel ( k_{\text{cat}} )</th>
<th>( k_{\text{cat}}(\text{H}_2\text{O}) )</th>
<th>( k_{\text{cat}}(\text{D}_2\text{O}) )</th>
<th>( \log(k_{\text{cat}}/K_{\text{app}}(\text{H}_2\text{O})) )</th>
<th>( k_{\text{cat}}/K_{\text{app}}(\text{H}_2\text{O}) )</th>
<th>( k_{\text{cat}}/K_{\text{app}}(\text{D}_2\text{O}) )</th>
<th>pK(<em>{a1}) of ( k</em>{\text{cat}}/K_{\text{app}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>100</td>
<td>2.34 ± 0.15</td>
<td>2.43 ± 0.42(^a)</td>
<td>8.2</td>
<td>1.07 ± 0.10</td>
<td>1.23 ± 0.20(^a)</td>
<td>6.3</td>
</tr>
<tr>
<td>Phenyl acetate</td>
<td>107</td>
<td>2.04 ± 0.28(^b)</td>
<td>2.32 ± 0.23(^c)</td>
<td>6.9</td>
<td>1.23 ± 0.17(^b)</td>
<td>1.46 ± 0.10(^a)</td>
<td>5.5</td>
</tr>
<tr>
<td>Isoamyl acetate</td>
<td>13</td>
<td>2.84 ± 0.70</td>
<td>5.8</td>
<td>1.26 ± 0.28</td>
<td>5.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( p )-Nitrophenyl acetate</td>
<td>8</td>
<td>2(^d)</td>
<td>5.4</td>
<td>1.93 ± 0.07</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl acetate</td>
<td>—</td>
<td>3.1</td>
<td>2(^d)</td>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( p )-Nitrophenyl diethylphosphate</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>6.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Unless otherwise noted, values of \( k_{\text{cat}} \) were observed at pH 8.5, 0.1 M NaCl, and are defined relative to an arbitrary value of 100 for the \( k_{\text{cat}} \) of acetylcholine. Values of \( k_{\text{cat}}/K_{\text{app}} \) are the observed second order rate constants (M\(^{-1}\) sec\(^{-1}\)) at pH 8.5, 0.1 M NaCl, assuming an actual value of \( k_{\text{cat}} \) of acetylcholine of 1.6 × 10\(^5\) sec\(^{-1}\) (see ref.13). Values of pK\(_{a1}\) were obtained in H\(_2\)O as outlined in the Materials and Methods. The error value for the D\(_2\)O effect is the square root of the estimated variance, determined for the ratio as outlined previously (22). \(^{a}\)Solvent contained 1.0 M NaCl at pH 8.5. \(^{b}\)pH stat assay. \(^{c}\)Spectrophotometric assay. \(^{d}\)An insufficient number of observations were made to statistically analyze these values.

Deuteration has been made for the pK values associated with the dephosphorylation of acetylcholinesterase (25). In 1.0 M NaCl significant binding of Na\(^+\) to the catalytic anionic site may be inferred from its competitive inhibition of acetylcholine hydrolysis. The R value at pH 8.5 for \( k_{\text{cat}}/K_{\text{app}} \) with acetylcholine in 1.0 M NaCl relative to 0.1 M NaCl is 0.26. This Na\(^+\) binding, however, blocks neither phenyl acetate binding (the corresponding R is 1.47) nor that of isoamyl acetate or methyl acetate (T. L. Rosenberry and E. Bock, unpublished observations); it also has only a slight effect on deacylation (R values at pH 8.5 for \( k_{\text{cat}} \) with acetylcholine and phenyl acetate in 1.0 M NaCl relative to 0.1 M NaCl are 0.90 and 0.85, respectively).

Detailed Analysis of the pH Dependence of \( k_{\text{cat}}/K_{\text{app}} \) for Neutral Acylating Agents. Although the apparent pK\(_{a1}\) values of 5.5 for phenyl acetate and 6.3 for acetylcholine in Table 1 coincide with general assignments for neutral and cationic acetic acid ester substrates by Krupka (20), a detailed investigation with a series of neutral acetic acid esters and with \( p \)-nitrophenyl diethylphosphate shows that such a dichotomized classification does not hold. In Fig. 2 the pH dependence of \( k_{\text{cat}}/K_{\text{app}} \) in the region of pK\(_{a1}\) for phenyl acetate and isoamyl acetate differs significantly from that of methyl acetate and \( p \)-nitrophenyl diethylphosphate. The precise pK\(_{a1}\) values are difficult to determine because the appropriate 1/R values do not increase linearly with [H\(^+\)]. Instead, the values of \( k_{\text{cat}}/K_{\text{app}} \) at low pH for all substrates studies are higher than those anticipated for linear inhibition by [H\(^+\)], an observation also made previously (20). Estimates of pK\(_{a1}\) from the initial slopes of 1/R versus [H\(^+\)] are included in Table 2. Methyl acetate, \( p \)-nitrophenyl acetate, and \( p \)-nitrophenyl diethylphosphate all give pK\(_{a1}\) estimates significantly greater than the 5.5 value for phenyl acetate and approach the 6.3 value for acetylcholine.

Deuteration Oxide Effects on \( k_{\text{cat}}/K_{\text{app}} \) and \( k_{\text{cat}} \). The values of \( k_{\text{cat}}/K_{\text{app}} \) and \( k_{\text{cat}} \) in D\(_2\)O relative to the corresponding values in H\(_2\)O are also given in Table 2. These values for acetylcholine as a function of pH are shown in Fig. 3. Because of the broad pH maximum shown by acetylcholinesterase, the deuteration oxide effects on the kinetic parameters observed at pH 8.5 should be little affected by deuterium isotope effects on the pK\(_{a1}\) values of enzyme catalytic groups. A deuteration oxide effect on \( k_{\text{cat}} \) of somewhat greater than 2 is observed both for acetylcholine and phenyl acetate for which \( k_{\text{cat}} \approx k_3 \), and for substrates for which \( k_{\text{cat}} < k_3 \). The observed effect on \( k_{\text{cat}} \) for phenyl acetate is consistent with a previous report of 2.3 (9). In contrast, deuteration oxide effects for \( k_{\text{cat}}/K_{\text{app}} \) vary from 1.1 for acetylcholine to 1.9 for \( p \)-nitrophenyl acetate. To our knowledge, only one

Fig. 2. The pH dependence of \( k_{\text{cat}}/K_{\text{app}} \) for several neutral acetic acid esters and for \( p \)-nitrophenyl diethylphosphate with acetylcholinesterase. R values for \( k_{\text{cat}}/K_{\text{app}} \) in 0.1 M NaCl were calculated for \( O \) phenylacetate; \( O, M \) isoamyl acetate; and \( A \) methyl acetate. Open symbols with these substrates represent R values from reciprocal plot slopes (see Materials and Methods), while filled symbols represent individual paired observations at a single substrate concentration far below \( K_{\text{app}} \) (\( * \) R values for second-order phosphorylation rates in 0.1 M NaCl with \( p \)-nitrophenyl diethylphosphate obtained as outlined in the Materials and Methods). The second order phosphorylation rate was 1.8 × 10\(^5\) M\(^{-1}\) sec\(^{-1}\) from data at 18 \( \mu \)M \( p \)-nitrophenyl diethylphosphate. Phosphorylation data obtained both with methyl acetate and with \( p \)-nitrophenyl acetate are included.

Fig. 3. Deuteration oxide effects on \( k_{\text{cat}}/K_{\text{app}} \) and \( k_{\text{cat}} \) for acetylcholine with acetylcholinesterase. Values were obtained as outlined in the Materials and Methods. At the time of the assay the solvent contained at least 98\% D\(_2\)O in all cases. The pH values were read directly from the pH meter, standardized in the usual way with buffers in H\(_2\)O. (O), H\(_2\)O; (●), D\(_2\)O.
other example of a deuterium oxide effect as low as 1.1 for either $k_{cat}$ or $k_{cat}/K_{app}$ for a serine hydrolase has been reported, and that concerns a series of very poor substrates for chymotrypsin (see ref. 26). While the number of substrates analyzed here is small, it is noteworthy that neutral substrates with deuterium oxide effects for $k_{cat}/K_{app}$ of less than 1.4 are associated with the lowest apparent $pK_{a1}$ values for $k_{cat}/K_{app}$.

**DISCUSSION**

The variation in the apparent $pK_{a1}$ from 5.5 to 6.3 among several acetic acid ester substrates is inconsistent with a formulation of Scheme 1 in which E and E-RX are in virtual equilibrium. Two formal ways of extending Scheme 1 to account for this variation may be proposed. In the first, protonation of either of two distinct groups in the free enzyme is postulated to affect activity by preventing substrate binding and/or blocking general acid-base catalysis. The affinity of cationic inhibitors for either the free or the acetylated enzyme is decreased by more than an order of magnitude with decreasing pH (20, 27). The apparent $pK_{a1}$ and $pK_{a1}'$ corresponding to these decreases are 6.2–6.3, the same as that for the pH dependence of $k_3$. From the coincidence of these $pK_a$ values plus further data on the effects of certain cationic ligands on deacetylation at low pH (20), Krupka (21) has proposed that an imidazole of $pK_{a1}$ 5.2–5.5 acts as a general base during acetylation and that a second imidazole of $pK_{a1}$ 6.3 both acts as a general base during deacetylation and, when protonated, blocks cation binding at the catalytic site. Alternatively, one could postulate a single general base catalyst of $pK_{a1}$ 5.2–5.5 in the free enzyme and $pK_{a1}'$ 6.3 in the acetyl enzyme; the $pK_{a1}$ of 6.3 in the free enzyme would correspond to the carboxylate group which defines the anionic site within the catalytic site. The pH dependence of betaine binding disputes this alternative postulate (20).

Proposals which invoke two distinct groups in the free enzyme have difficulty in explaining the current data. The variation in the apparent $pK_{a1}$ values among neutral substrates could be explained by postulating that protonation of the $pK_{a1}$ 6.3 group reduces the binding of some, but not all, neutral substrates. Low deuterium oxide isotope effects observed for $k_{cat}/K_{app}$ with certain substrates also would require a postulate of a variable deuterium oxide effect on substrate binding.

In view of these rather arbitrary postulates, it is of interest to consider a second formal extension of Scheme 1 in which the influence of kinetic rate constants alters the actual $pK_a$ value of a single catalytic group of $pK_{a1}$ 6.3 in the free enzyme. If virtual equilibrium of E and E-RX in Scheme 1 does not obtain ($k_2 > k_{-1}$), then $k_{cat}/K_{app} \approx k_1$ (see refs. 28 and 13). Under this condition the bimolecular rate constant $k_1$, not the general base catalysis step $k_2$, becomes rate-limiting at low substrate concentrations. As shown by Renard and Fersht (28), in this case the apparent $pK_{a1}$ of a basic group required for $k_2$ decreases by an amount directly related to $k_2/K_{-1}$. Furthermore, if $k_1$ becomes rate-limiting, little or no deuterium oxide effect would be expected. Although this formulation can account nicely for the low deuterium oxide effect observed here for acetylcholine, for which the high $k_{cat}/K_{app}$ value (Table 2) already suggests that $k_1$ is rate-limiting (13), it fails to explain similar observations in other substrates with much lower $k_{cat}/K_{app}$ values. Phenyl acetate and isovaleryl acetate give apparent $pK_{a1}$ values below 6.3 and show low deuterium oxide effects on $k_{cat}/K_{app}$ and carbamoylating agents, for which $k_{cat}/K_{app}$ values are some $10^4$ times smaller than those for acetic acid esters, show an analogous variation in $pK_{a1}$ values (29). It would appear that kinetic perturbations of $pK_{a1}$ would have to occur within a mechanism more complex than Scheme 1, one which permits virtual equilibrium of at least the initial Michaelis complex. Such a mechanism is presented in Scheme 2.

Scheme 2, which focuses on enzyme acylation, extends the simpler mechanism in Scheme 1 by introducing the intermediate (E-RX)'. In particular, it may be shown (see Appendix) that if general base catalysis by a group with an actual $pK_{a1}$ of 6.3 occurs at $k_2$ subsequent to the rate-limiting formation of (E-RX)' (i.e., $k_2 > k_{-1}'$), then the apparent $pK_a$ for $k_{cat}/K_{app}$ is less than 6.3. In this case (E-RX)' is a second enzyme-substrate intermediate formed by a conformational change of the initial Michaelis complex and is identical to the induced-fit complex proposed by Koshland (30). Induced-fit as a pre-equilibrium process has been considered likely for a number of enzymes (30) including acetylcholinesterase (31, 32; see ref. 13), but the author is not aware of other proposals of rate-limiting induced-fit in which the rate of the limiting conformational change is substrate dependent.

According to Eq. 2A derived from the rate-limiting induced-fit model, the apparent $pK_a$ for $k_{cat}/K_{app}$ is a function of rate constants which may be expected to vary for each acetylating agent. This prediction is consistent with observations in Table 2. Furthermore, if $k_1' \neq k_0'$, the inhibition of $k_{cat}/K_{app}$ may be nonlinear in [H$^+$], again as observed. Variations in this apparent $pK_a$ have been reported previously for neutral acetylating (27), carbamoylating (29), and phosphorylating agents (see ref. 13), all of which appear to utilize the same catalytic pathway (12). Phosphorylating agents are of particular interest in that, unlike other substrates and acetylating agents, second-order enzyme phosphorylation rates depend significantly on the quality of the leaving group and display positive rho values (see ref. 32). Consequently the rate-limiting step according to Scheme 2 would appear to be the general base-catalyzed step $k_2$, and any induced-fit step would occur in a prior equilibrium. In this case, the apparent $pK_a$ should be the actual $pK_a$ of the catalytic group. The $pK_a$ for second-order phosphorylation for one phosphorylating agent, p-nitrophenyldiethylphosphate, was observed to be 6.1 (Fig. 2, Table 2). This $pK_a$ approaches the highest $pK_a$ values observed with acetic acid esters and thus supports the idea that apparent $pK_a$ values below 6 are kinetically perturbed. However, the inhibition is again non-linear in [H$^+$], a complication not anticipated by Scheme 2 for an agent for which $k_2$ is presumably rate-limiting at all pH values. Such non-linearity may arise from the protonation of a second active site group which interacts with the $pK_{a1}$ 6.3 group or from the intervention of an acid-catalyzed pathway at low pH, complexities beyond the scope of Scheme 2.

In Fig. 4 a schematic visualization is offered as a summary of the induced-fit model in Scheme 2. Rate-limiting induced-fit obtains at low substrate concentrations with substrates for which $k_2 > k_{-1}'$. Under this condition at high pH, $k_{cat}/K_{app} \approx k_1'/K_1$ (Eq. 2A); although $k_1'$ is not well de-
fined, both it and $K_s$ seem likely to involve smaller deuterium oxide effects than a step involving general acid-base catalysis. Phenyl acetate and isoamyl acetate show both this reduced deuterium oxide effect as well as the greatest deviation in apparent $pK_a$ below 6.3; they thus satisfy two important criteria for rate-limiting induced-fit. In contrast, corresponding deuterium oxide effects of about 2 are observed for substrates which have a pH dependence of $k_{cat}/K_{app}$ similar to that for second-order phosphorylation with p-nitrophenyldiethylphosphate. The rate-limiting step for these substrates thus is consistent with general acid-base catalysis.

It is noteworthy that substrates rate-limited by induced-fit at low substrate concentrations may still be rate-limited by general base catalysis at substrate saturation if $k_1' > k_2$ in Scheme 2 (13). Indeed, $k_{cat}$ for isoamyl acetate shows a large deuterium oxide effect consistent with this formulation.

**APPENDIX**

According to Scheme 2 with the assumption of rapid equilibrium both among E, EH, E-RX and EH-RX and between (E-RX)' and (EH-RX)' and $k_{cat}/K_{app}$ is given by Eq. 2A.

$$\frac{k_{cat}}{K_{app}} = \frac{k_k k_1' \left( 1 + k_{cat}[H^+] \right)}{K_s}$$

[2A]

In Eq. 2A, $K_s = k_{-1}/k_1$. Eq. 2A is formally analogous to that arising from the simpler nonequilibrium mechanism considered by Renard and Fersht (28). An illustrative simplification of Eq. 2A considered by these authors assumed that $k_1' = k_{10}$. If it is further assumed here that $K_s = K_s'$, Eq. 3A obtains.

$$\frac{k_{cat}}{K_{app}} = \frac{k_k k_1'}{K_s'} \left( 1 + k_1' \frac{[H^+]}{K_s} \right)$$

[3A]

The observed $K_s$ in the case of Eq. 3A is given by Eq. 4A.

$$K_s'(observed) = \frac{1 + k_0/[k_{cat}/K_{app}]}{K_s}$$

[4A]

Hence the actual $K_s$ is perturbed by kinetic rate constants to the extent that $k_2 > k_{-1}'$. A qualitatively similar conclusion can be drawn from the general case in Eq. 2A in which $[k_{cat}/K_{app}]^{-1}$ is not necessarily linear in [H$^+$].

This investigation was supported, in part, by the U.S. Public Health Service Grants NS-03504-13 and NS-11766-01, by the National Science Foundation Grant NSF-CB-40055, and by the New York Heart Assoc. Inc. The careful technical assistance of Mr. E. Bock was an important contribution to this study.