Acetyl-Coenzyme A : Arylamine N-Acetyltransferase

ROLE OF THE ACETYL-ENZYME INTERMEDIATE AND THE EFFECTS OF SUBSTITUENTS ON THE RATE*

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SUMMARY

The reactions of p-nitroacetanilide and p-nitrophenyl acetate with aromatic and aliphatic amines catalyzed by a partially purified preparation of acetyl-coenzyme A:arylamine acetyltransferase follow "ping-pong" kinetics. At a given concentration of p-nitroacetanilide the identical maximum velocities observed for five substituted anilines, semicarbazide, hydroxylamine, and hydrazine, suggest that these reactions proceed through the rate-determining formation of a common acetyl-enzyme intermediate which reacts with the acceptor amine in a fast step. The maximum velocities are directly proportional to the p-nitroacetanilide concentration. p-Nitrophenyl acetate is the most active acyl donor substrate known for the enzyme, with a maximum velocity approximately 140-fold larger than that of acetyl-CoA. With weakly basic anilines the maximum velocity is different for different acyl acceptors, suggesting that with these substrates the reaction of the acetyl-enzyme intermediate with the acceptor amine is rate determining and that the saturation with increasing amine concentration represents binding of the acceptor amine to the acyl-enzyme. With strongly basic anilines the maximum velocities and the $K_m$ for p-nitrophenyl acetate are identical, within experimental error, for all anilines; this is interpreted as evidence for a change to rate-determining acylation of the enzyme with these more reactive nucleophiles. The increase in reactivity with increasing aniline basicity suggests the development of a partial positive charge on the attacking nitrogen atom, which may be accompanied by a relatively small degree of proton removal by a general base catalyst. It is suggested that the wide variation in the sensitivity of enzyme-catalyzed acyl transfer reactions to the basicity of the nucleophile or leaving group reflects different degrees of proton removal or addition by general acid-base catalysts in the transition state.

Acetyl-coenzyme A:arylamine N-acetyltransferase (EC 2.3.1.5), the second component of the sulfanilamide acetylation system discovered by Lipmann and coworkers, catalyzes the transfer of an acetyl group from the thiol ester of acetyl-CoA to a variety of substituted anilines and to several nonaromatic amines (Equation 1) (1, 2). It has been suggested that catalysis of this acetyl transfer and of an analogous acetyl transfer (Equation 2) from substituted acetanilides (such as acetyl-4-aminoazobenzene-4'-sulfonic acid)

$$\text{AcSCoA} + H_2NR \rightarrow \text{AcNHR} + HSCoA \hspace{1cm} (1)$$

$$\text{AcNHR} + H_2NR \rightarrow \text{AcNHR} + H_2N\text{ArX} \hspace{1cm} (2)$$

to anilines and other amines (including labeled AABS), to give an exchange reaction, proceeds through the formation of an acetyl-enzyme intermediate (3), but the possibility of a direct acetyl transfer from the donor to the acceptor has not been ruled out. A stronger indication for the formation of an acetyl-enzyme intermediate is found in the "ping-pong" kinetics, with characteristic inhibition patterns by the initial product, for the reaction (4, 5), but again the evidence is not conclusive. The pigeon liver enzyme is reversibly inactivated by sulfhydryl reagents or by oxidation, but related mammalian enzymes are more resistant to such inactivation (2, 6). The experiments reported here were carried out in an attempt to obtain further information regarding the possible existence of an acetyl-enzyme intermediate in the reaction and to examine the charge distribution in the transition state of the catalyzed reaction by determining the effect of polar substituents on the reaction rate under defined conditions. A previous study by Jacobson had indicated that the reaction rate increases with electron-donating substituents on the acceptor aniline and with electron-withdrawing substituents on the donor acetanilide, but it was not determined whether the enzyme was saturated with substrate, or whether the formation or breakdown of an acetyl-enzyme intermediate was rate determining under the limited range of experimental conditions examined (7, 8). Some of the results described here have been reported in a preliminary communication (9).

EXPERIMENTAL PROCEDURE

Materials—Aniline, other amines, and acetyl donors, with the exception of acetyl-CoA, were purified by sublimation, recrystallization, or distillation from zinc dust at reduced pressure. Phenylendiamine dihydrochloride was recrystallized from hydrochloric acid-stannous chloride (10). AABS and acetyl-

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1 The abbreviations used are: AABS, 4-aminoazobenzene-4'-sulfonic acid, PNPA, p-nitrophenyl acetate.
AABS were kindly given by Dr. S. Bessman. Anilines were stored under argon. Acetyl-CoA was obtained from P-L Biochemicals or Calbiochem, 95% purity. Acetonitrile was redis-tilled.

Acetyl-CoA: arylamine N-acetyltransferase was partially purified (11) to approximately the same specific activity obtained by Jacobson (7); the purification procedure will not be described here in detail. Frozen pigeon livers, obtained from the Palmetto Pigeon Plant (Sumter, South Carolina), were thawed, minced, and homogenized. Inactive protein was precipitated from the extract with protamine sulfate and the enzyme was concentrated with ammonium sulfate (0 to 65%). These steps were carried out at the New England Enzyme Center. The enzyme was purified by chromatography on DEAE-cellulose, removal of inactive protein with hydroxylapatite, chromatography on Sephadex G-100, fractionation with alumina gel C(7), and chromatography on QAE-Sephadex. Some increase in stability during purification steps and on storage was obtained by the use of 10% ethylene glycol and by reversibly blocking sulphydryl groups by treatment with 3,3'-dithiodipropionic acid. Enzyme activity was found to be inhibited by p-chloromercuribenzoate (5 x 10^-4 M) and by 5,5'-dithiobis(2-nitrobenzoic acid) (0.015 M) and was reactivated by 0.01 M dithiothreitol. The enzyme was 54% inactivated by treatment with 0.13 M dithiodipropionic acid in 0.05 M Tris, pH 9, for 46 hours at 0°C and 57% recovery of activity was obtained after incubation for 20 min with 0.01 M dithiothreitol, 0.01 M EDTA, in 0.1 M Tris, pH 8. The enzyme activity was stable at -17°C in the presence of 0.01 M potassium phosphate, pH 7.8, 0.001 M EDTA, 0.015 M mercaptoethanol in 10% ethylene glycol for more than 6 months (freezing in more concentrated thiol or in 0.01 M dithiothreitol causes inactivation). Upon electrophoresis in 7% acrylamide gel at pH 7.5 and 8.9 (12, 13), the enzyme activity with p-nitroanilide and AABS was found to move ahead of the major protein band. The activity with PNPA as acetyl donor was found to have the same mobility as that with p-nitroanilide.

Methods—Reaction rates were followed spectrophotometrically at 25°C by measuring the release of p-nitroaniline (ε 11,220, 400 nm (7)) from p-nitroacetanilide; AABS (ε 10,350 at pH 7.4 to 8.5, 460 nm) from acetyl AABS (ε 1,650, pH 7.4 to 8.5, 460 nm); or p-nitrophenol (ε 13,400, pH 7.4, 400 nm) from PNPA. The enzyme was activated by a prior incubation in the presence of 0.01 M dithiothreitol, 0.01 M EDTA, and 0.01 M potassium phosphate, pH 7.4, for 20 to 30 min at 25°C. This preliminary incubation was usually carried out with a concentrated enzyme solution in 10% ethylene glycol in order to minimize the concentration of thiol in the final reaction mixture. For experiments with PNPA the enzyme was further diluted and stored under argon in a series of tubes fitted with serum caps. A new tube was used about every 30 min and the activity of the enzyme was monitored with a standard reaction mixture with aniline as the acyl acceptor.

The observed reaction rates were corrected for the nonezymatic reaction of PNPA with the remaining dithiothreitol and the acceptor aniline. Reaction mixtures contained 0.01 M potassium phosphate, pH 7.4, 0.01 M EDTA, potassium chloride to give an ionic strength of 0.3, PNPA (added in 20 to 50% acetonitrile), aniline, acetonitrile to a final concentration of 5%, and enzyme. The reactions were followed in 3.5-ml cuvettes with a 1.0-cm or a 0.2-cm path length, maintained with a quartz spacer. The shorter path length was used for anilines with PK < 4. The reaction rate was shown to be linear with respect to enzyme concentration over a 40-fold range. The activity is expressed in terms of enzyme units (e.u.) defined as the amount of enzyme which gives an absorbance change of 0.035 per min in a final volume of 3.0 ml, corresponding to 7.9 nanomoles per min per ml, in the standard assay containing 10^-3 M PNPA and 0.01 M aniline. The partially purified enzyme has a turnover of 63 nanomoles per min per g under these conditions. Solutions of anilines were stored in test tubes fitted with serum caps and flushed with argon; a fresh tube was opened about every 30 min. A concentrated solution of p-phenylendiamine dihydrochloride was 90% neutralized with potassium hydroxide just before use, approximately every 30 min. Rate measurements were usually obtained from linear changes in absorbance over a period of 2 min after the first 30 sec. The reaction rate was initiated by the addition of PNPA, which was added immediately after the addition of enzyme.

The rate of the enzyme-catalyzed reaction of acetyl-CoA with aniline was followed by measuring the rate of absorbance change at 412 nm in the presence of 10^-4 M Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid), product ε 13,600, 412 nm (14)). The observed rates were corrected for a small absorbance change that occurred in the absence of aniline. The reaction rate is nonlinear under these conditions because of inhibition of the enzyme by Ellman's reagent. The reaction rates were corrected for this inhibition of 6% and 23% during the first and second minutes of reaction, respectively, as measured in a standard assay mixture containing 10^-4 M PNPA and 0.01 M aniline.

In the presence of p-nitroacetanilide the enzyme catalyzes the appearance of p-nitroaniline in the absence of aniline at approximately 15% of the rate observed in the presence of aniline; this activity ratio remained approximately constant during the purification. No correction was made for this hydrolysis because it does not affect the relative values of the maximal rates observed in the presence of different anilines and has only a relatively small effect on the K_m values; if the rates are corrected for hydrolysis the K_m values are increased by approximately 20%. The rate measurements with p-nitroanilide were generally carried out at relatively high aniline concentrations in order to obtain accurate maximum velocities. It is possible that the hydrolytic reaction is suppressed when aniline is present to act as an acyl acceptor. No hydrolytic reaction was observed with the much more active substrate PNPA as acetyl donor.

RESULTS

Reactions with p-Nitroacetanilide—Double reciprocal plots of the enzyme-catalyzed rate of acetyl transfer as a function of the concentration of amine acceptor at several concentrations of p-nitroanilide as acetyl donor give parallel line, ping-pong, kinetics (Fig. 1), confirming previous results with other substrates (4, 5). At a constant concentration of 5 x 10^-3 M p-nitroanilide, the maximum rates of acetyl transfer are identical at saturating concentrations of five different substituted anilines and three "a-effect" amines, semicarbazide, hydroxylamine, and hydrazine (Table I). Approximate K_m values for these amine acceptors, also shown in Table I, are very small for uncharged anilines and several orders of magnitude larger for p-aminobenzoate and the other amine acceptors. Both the V_m and the K_m values for the amines depend on the concentration of the acetyl donor, as a consequence of the ping-pong kinetics, but with an increase in p-nitroacetanilide concentration the maximum velocity increases to the same value for the reaction with hy-
droxylamine as for the reaction with aniline, in spite of the very different $K_m$ values for these amines (Fig. 2; note the different abscissa scales for the two amines). No evidence for a saturation of the enzyme with respect to p-nitroacetanilide concentration was obtained with aniline or hydroxylamine as acetyl acceptors; it is possible that the apparent saturation observed previously with $3 \times 10^{-5}$ M AABS and $6 \times 10^{-5}$ M aniline (7, 16) is a consequence of a change in rate-determining step or a specific effect of AABS.

Reactions with Other Acetyl Donors Since the common $V_{\text{max}}$ values for different amines in the reaction with p-nitroacetanilide indicated that at saturating amine concentrations there is a common rate-determining step in which the amine is not involved, presumably the formation of an acetyl-enzyme intermediate, we examined other acetyl donors in the hope of finding one which would form the acetyl-enzyme sufficiently rapidly that the reaction with the amine would become rate determining. Under these conditions the observed $V_{\text{max}}$ would be different for different amines and would serve as a measure of the nucleophilic reactivity of the bound amine in the enzyme-substrate complex.

$N,S$-Diacetylmercaptoethylamine, an analogue of acetyl-CoA, was found to be an active, but poor, substrate for the enzyme. The activity with 5 mM $N,S$-diacetylmercaptoethylamine is the same as that with 0.5 mM p-nitroacetanilide, with 0.5 mM AABS as acetyl acceptor.

$p$-Nitrophenyl acetate, in which the nitrogen atom of p-nitroacetanilide is replaced by oxygen, was found to be the most active substrate yet reported for the acetyltransferase, with a maximal activity more than 100-fold larger than that of acetyl-CoA (Table II). The $K_m$ value for PNPA is 10 to 20 times larger than that of acetyl-CoA, however, so that at approximately 2 mM substrate the rate difference is only 30-fold. The enzyme-catalyzed acetylation of aniline by PNPA is competitively inhibited by $10^{-4}$ M acetyl-CoA, which gives 2% inhibition at 0.4 mM PNPA and 2% inhibition at 1.5 mM PNPA. The $K_m$ value for acetyl-CoA is within the wide range of previously reported values (4, 5, 17).

![Fig. 1](image1.png)

**Fig. 1.** The effect of aniline and p-nitroacetanilide concentrations on the rate of enzyme-catalyzed acetyl transfer at pH 8.0, 25°. The velocity is expressed as $\Delta A_400$ per ml of enzyme per min $\times 10^3$. 

![Fig. 2](image2.png)

**Fig. 2 (left).** The extrapolation to identical maximal velocities of the reaction rates of aniline (■ and ○, lower scale) and of hydroxylamine (▲ and △, upper scale) at two concentrations of p-nitroacetanilide at pH 8.0, 25°. The velocity is expressed as $\Delta A_400$ per ml of enzyme per min $\times 10^3$.

![Fig. 3](image3.png)

**Fig. 3 (center).** The effect of substrate concentrations on the rate of reaction of m-fluoroaniline with PNPA catalyzed by transacetylase at pH 7.4, 25°. The velocity is expressed as $\Delta A_{400}$ per e.u. per min $\times 10^3$. 

![Fig. 4](image4.png)

**Fig. 4 (right).** The effect of substrate concentrations on the rate of the reaction of p-phenylenediamine with PNPA catalyzed by transacetylase at pH 7.4, 25°. The velocity is expressed as in Fig. 3.

| Amine acceptor | $pK_a$ | Ratio of $V_{\text{max}}$ (amine) to $V_{\text{max}}$ (aniline) | $K_m$ (approx) |
|----------------|-------|-------------------------------------------------|----------------|
| NH$_2$NH$_2$   | 4.6   | 0.97                                            | 1.6 $\times 10^{-4}$ |
| NH$_2$OH       | 6.0   | 0.97                                            | 1.6 $\times 10^{-4}$ |
| NH$_2$NH$_2$   | 8.1   | 0.97                                            | 2.2 $\times 10^{-4}$ |

*Values are taken from Reference 15.

| $[\text{PNPA}]_{\text{M}} \times 10^3$ | $[\text{pNPA}]_{\text{M}} \times 10^3$ |
|---------------------------------------|---------------------------------------|
| 0.000                                | 0.5                                   |
| 0.025                                | 0.067                                 |
| 0.050                                | 2.0                                   |
| 0.100                                | 1.34                                  |
| 0.200                                | 2.0                                   |
| 0.400                                | 2.0                                   |

Table I

Kinetic constants for acetyl transfer from $5 \times 10^{-4}$ M p-nitroacetanilide at 25° and pH 8.0

The reaction mixtures contained $5 \times 10^{-4}$ M p-nitroacetanilide, 5% acetonitrile, 0.1 M Tris, pH 8, 0.01 M EDTA, 0.01 M dithiothreitol, potassium chloride to give ionic strength 0.3 and enzyme in a final volume of 3.0 ml.
Comparison of kinetic constants for acetyl-CoA and p-nitrophenyl acetate at saturating aniline concentrations

The reaction mixtures contained 0.01 M potassium phosphate, pH 7.4, 0.001 M EDTA, 5% acetonitrile, 0.1 mM Ellman's reagent, aniline (10, 7.5, or 5 mM), acetyl-CoA (0.5, 1.0, 2.0, or 2.5 mM), and enzyme in a volume of 1.0 ml at 25°C.

| Acetyl donor          | $V_{\text{max}}$ | $K_m$ of the acetyl donor | Ratio of $V_{\text{max}}$ (PNPA) to $V_{\text{max}}$ (acetyl-CoA)$^a$ |
|----------------------|------------------|--------------------------|-------------------------------------------------------------|
| Saturating acetyl-CoA | 0.60             | 34                       | 140                                                         |
| Saturating PNPA      | 5.00             | 1.1                      | 10                                                          |
| 2.2 mM acetyl-CoA     | 0.49             | 1.0                      | 10                                                          |
| 2.0 mM PNPA          | 0.48             | 1.0                      | 10                                                          |

$^a$ Maximum velocity with respect to the concentration of aniline.

Phenyl acetate is less active than PNPA as an acetyl donor. In the presence of 0.1 mM AABS the $V_{\text{max}}$ for PNPA was found to be 5.7 times larger than that of phenyl acetate in 0.1 M potassium phosphate buffer, pH 7.4. Under these conditions the observed rate was independent of PNPA concentration from 0.5 to 2.0 mM and independent of phenyl acetate concentration from 2.0 to 3.0 mM. Acetylindazolone at 2 mM was found to be unreactive as an acetyl donor to 10 mM AABS at an enzyme concentration equal to 10 times that which gave a rate of 13.8 µmols per min at 1.0 mM PNPA and 0.1 mM AABS.

Reactions with p-Nitrophenyl Acetate—The more weakly basic anilines were found to react at different, relatively slow maximal rates with PNPA as the acetyl donor, thereby establishing that the aniline is involved in the rate-determining step and that acetylation of the enzyme by PNPA is not rate determining for these substrates. The kinetic constants for these substrates were determined without difficulty, as shown in Fig. 3 for the reaction of m-fluoroaniline. The reactions of the more basic anilines also clearly follow ping-pong kinetics, but accurate determination of the maximum velocities is difficult because of the considerable extrapolation to saturating substrate concentration necessitated by the limited solubility of PNPA; the data for p-phenylenediamine are shown in Fig. 4 as an example. These anilines were examined in a number of experiments and in every case a non-zero intercept on the ordinate was observed, but the absolute value of the intercept was difficult to determine from a single experiment. In Fig. 5 the data from the experiments with all anilines that have pKa values greater than 4 are plotted on the same graph; the range of observed values is shown by the bars at each concentration of PNPA. A single line fits the data for all the anilines within experimental error and gives the kinetic constants for PNPA which are summarized in Table III along with the individual kinetic constants obtained for these and the other substituted anilines.

Substrate inhibition was observed at aniline concentrations of approximately 0.01 M or more and was more marked at the lower concentrations of PNPA.

**TABLE III**

Kinetic constants for reactions of PNPA and substituted anilines catalyzed by acetyltransferase at pH 7.4, 25°C

| Aniline | $V_{\text{max}}$ | $K_m$ of PNPA | $K_m$ of aniline | Ratio of $V_{\text{max}}$ to $V_{\text{max}}$ (PNPA) |
|---------|------------------|---------------|-----------------|-----------------------------------------------------|
| X = p-CN | 1.74             | 2.5           | 0.49            | 1.1                                                 |
| O       |                  |               |                 | 2.4                                                 |
| p-CH$_3$C | 2.19             | 2.0           | 0.89            | 1.7                                                 |
| O       |                  |               |                 | 1.2                                                 |
| p-C$_2$H$_4$O | 2.51           | 7.7           | 0.86            | 1.1                                                 |
| m-F     | 3.41–3.57        | 8.6           | 1.18            | 6.3                                                 |
| m-CF$_3$ | 3.49             | 2.4           | 0.53            | 2.7                                                 |
| m-Cl    | 3.98             | 5.0           | 5.2             | 0.9                                                 |
| Hydrogen| 4.6              | 11.4          | 18              | 6.2                                                 |
| m-CH$_3$ | 4.67             | 137           | 14.38           | 17                                                  |
| m-CH$_2$ | 5.07             | 80            | 8.78            | 2.3                                                 |
| m-CH$_2$O | 5.34             | 45            | 4.38            | <1.0                                                |
| p-NH$_2$ | 6.3              | 54            | 5.28            | 1.8                                                 |

$^a$ pKa values (determined at 25°C, $I = 0$) were selected from Reference 15.

$^b$ Value from individual plot (least squares).

$^c$ Value from least squares line through the composite plot of Fig. 5.
Acetyl-CoA: Aminylamine N-Acetyltransferase

The relationship between the maximum velocity and the basicity of the aniline for the reactions of PNPA with a series of substituted anilines catalyzed by acetyltransferase at pH 7.4, 25°C. For anilines of pK > 4 the \( V_{\max} \) values determined from the collected data (Fig. 5) and from least squares plots of the data for individual anilines (Table III) are shown.

The much faster reactions observed with anilines of pK less than 4 exhibit a different maximum velocity for each aniline (Table III, Fig. 6). This leads to two conclusions. (a) The deacylation step, in which the aniline reacts with the acetyl-enzyme intermediate, must be rate determining. (b) There must be a binding site for anilines, since if the anilines reacted with the acetyl-enzyme in a simple second-order reaction, with no binding, no maximum velocity would be observed.

The acetyltransferase provides a particularly clearcut example of a situation in which an observed Michaelis constant may reflect either a binding of substrate or a change in rate-determining step (Fig. 7). When deacylation is rate determining, the saturation with increasing aniline concentration reflects aniline binding and the \( K_m \) is equal to \( K_s \), the true dissociation constant (Fig. 7, upper line). However, when the acylation step is slow, as in the p-nitroacetanilide reactions, the rate initially increases with increasing aniline concentration, as in the former case, but soon must level off because the acylation step becomes rate limiting and the over-all rate cannot increase further (Fig. 7, lower line).

Under these conditions the "saturating" is a consequence of a change in rate-determining step, from rate-determining deacylation at very low aniline concentration to rate-determining acylation at higher aniline concentrations, and the \( K_m \) value is a kinetic constant that indicates the aniline concentration at which the change in rate-determining step is taking place. For example, the true dissociation constant, \( K_s \), for m-fluoracilnline, measured with PNPA as acetyl donor, is \( 6.3 \times 10^{-4} \) M, whereas the kinetic \( K_m \), with \( 5 \times 10^{-4} \) M p-nitroacetanilide as acetyl donor, is \( 3.4 \times 10^{-4} \) M, more than 1000 times smaller (Tables I and III). With rate-determining acylation both the \( V_{\max} \) and the \( K_m \) increase with increasing acetyl donor concentration and acetylation rate and are, in fact, directly proportional to the acetylation rate.
This is shown in Fig. 2 for the reactions of aniline and hydroxylamine in the presence of two concentrations of p-nitroacetanilide as acetyl donor and is shown schematically by the middle line of Fig. 7. No leveling off of the rate with respect to p-nitroacetanilide concentration was observed at concentrations up to $10^{-3}$ M, near the solubility limit, so that the $V_{max}$ and $K_m$ values for different amines are directly proportional to p-nitroacetanilide concentration over the accessible concentration range for this acetyl donor.

If it is assumed that the adsorption and desorption steps are fast, and the results are most easily explained with this assumption, the apparent Michalis constants for the acyl donor and acceptor, respectively, are given in Equations 5 and 6, based on the two-step reaction mechanism of Equations 7 and 8. The

$$K_m = K_k \frac{k_2}{k_1}$$

and

$$K_m' = K'_k \frac{k_1}{k_2}$$

above situation may be described in terms of these equations by the statement that for the PNPA reactions with weakly basic anilines $k_1 > k_2$, so that $K_m' = K'_k$, for the acceptor amine, and for the p-nitroacetanilide reactions $k_2 > k_1$, so that $K_m = K_k$ for the acetyl donor. Conversely, the observed $K_m$ value for the acceptor amine is dominated by the ratio of rate constants $k_1/k_2$ for the p-nitroacetanilide reactions, for which $k_2 > k_1$ and $K'_m = K'_k k_2/k_1$, and the $K_m$ for PNPA is dominated by the inverse ratio of rate constants when $k_1 > k_2$ and $K_m = K_k k_1/k_2$.

The $K_m$ values for substituted anilines, with the exception of p-aminobenzoate, decrease progressively with increasing basicity of the amine in the p-nitroacetanilide reaction series (Table I). Since the observed $K_m$ values for this reaction include the rate constants for reaction of the bound amine as well as the dissociation constant of the amine, this trend is consistent with an increase in reactivity of the bound amine with increasing basicity, in accord with the known increase in chemical reactivity of anilines and other amines with increasing basicity in acyl transfer reactions (22-25). A similar situation has been described by Kirsch and Igelstrom (19) for the hydrolysis of a series of substituted phenyl esters of benzoylcarbonylglycine by papain, in which the decrease in the observed $K_m$ values is correlated with an increase in chemical reactivity, as measured by the rate of alkaline hydrolysis, and presumably with the rate of acylation of enzyme by the esters. The high $K_m$ value for p-aminobenzoate reflects an unfavorable interaction of this amine with the active site which may be attributed to its negatively charged carboxyl group; a low reactivity of p-aminobenzoate, examined at a single concentration, has been noted previously (7). The $K_m$ values for the nonaromatic amines semicarbazide, hydroxylamine, and hydrazine are several orders of magnitude larger than those for the anilines. This means that these compounds, all of which are $\alpha$-effect compounds with a high chemical reactivity toward acyl compounds (25, 26), and two of which are more basic than any of the anilines examined, are poor acyl acceptors in the reaction catalyzed by acetyl transferase. It may be concluded that binding to the active site of the enzyme brings about a large increase in the reaction rate of anilines, the specific substrates, and that these nonaromatic amines either react from solution or interact weakly and/or ineffectually with the amine binding site. Binding of anilines to the donor-acceptor binding site is also manifested in the substrate inhibition that is observed at high aniline and low acyl donor concentrations.

Although the maximum velocities for the reactions of PNPA with weakly basic anilines vary over a wide range, there is no significant difference in the maximum velocities for anilines with $pK$ values over 4.0 (Fig. 5). The simplest interpretation of this result is that it represents a change in rate-determining step such that acylation of the enzyme by PNPA becomes rate determining for the more basic anilines; i.e., the weakly basic anilines react with the acetyl enzyme relatively slowly, so that acylation is fast relative to deacylation, but with the more reactive and more basic anilines the rate of acylation is no longer able to keep up with the rate of deacylation, even with PNPA as acetyl donor, and the acylation step becomes rate determining to give a common maximum velocity for these anilines. This could be tested directly if a more reactive acyl donor than PNPA could be examined, but it will be difficult to find such a donor because the non-enzymatic reaction of more reactive acylating agents, such as 2,4-dinitrophenyl acetate, interferes with measurements of the enzymatic reaction rate. However, the hypothesis is supported by the fact that the $K_m$ as well as the $V_{max}$ values for PNPA are identical, within experimental error, for all of the strongly basic anilines examined (Fig. 5). This is not the case for the weakly basic anilines; in these reactions the smaller observed $K_m$ values for PNPA include the rate constant for the acylation step (Table III). This suggests that with the strongly basic anilines the observed $K_m$ value for PNPA is a true dissociation constant that is independent of the nature of the acceptor amine, under conditions in which acylation is rate determining.

The chemical nature of the acetyl enzyme intermediate has not been established, but the fact that the enzyme requires a free sulfhydryl group suggests that it may be a thiol ester. This assignment is supported by the results of preliminary experiments which show that the enzyme is completely protected against inhibition by iodoacetamide in the presence of concentrations of PNPA well below the $K_m$ for PNPA, conditions in which the nucleophilic group in the enzyme is expected to be protected by acetylation, and that this protection by PNPA is removed upon the addition of anilines, which are expected to decrease the steady state concentration of acetyl enzyme and thereby free the nucleophilic group for reaction with inhibitors.*

The acetyl group transfer reactions catalyzed by the acetyl transferase are closely analogous to the alkyl group transfer reactions catalyzed by thiaminase I. The latter reaction also proceeds through an intermediate composed of the alkyl and group being transferred and either the formation or the breakdown of the intermediate can be made the rate-determining step by varying the nature and the concentration of the alkyl group donor and acceptor, so that under conditions in which the reaction of one substrate is rate determining, the observed rate is independent of the concentration and nature of the other

* F. Huneeus and W. P. Jencks, unpublished experiments.
substrate (27). It has also been suggested that a nonlinear structure-reactivity relationship for the thiaminase-catalyzed reactions of a series of substituted anilines, based on measurements at a single concentration of each substrate, may reflect a change in rate-determining step with changing aniline structure (28).

Structure-Reactivity Relationships—Substitution of the amino nitrogen atom of p-nitroacetanilide by oxygen, to give p-nitrophenyl acetate, causes a large increase in enzymatic, as well as chemical, reactivity, so that PNPA is by far the most active acetyl donor known for acetyltransferase. PNPA appears to be a true substrate for the enzyme, and not simply a chemical acetylating agent which reacts from solution, because (a) the saturation with respect to PNPA concentration in the reactions with basic anilines suggests that PNPA binds to the active site and (b) acetylimidazole, an active acetylating agent with a very similar acetyl group potential and an even higher chemical reactivity compared with PNPA (29, 30) is inactive as a substrate.

Phenyl acetate, with a leaving group that is three orders of magnitude less acidic than that of PNPA, is a much poorer substrate than PNPA. The 5.7-fold smaller $V_{\text{max}}$ for phenyl acetate compared with PNPA in the reaction with AABS means that acylation must be rate determining in the phenyl acetate reaction. Acylation is not rate determining for PNPA under these conditions, however, because a much faster rate is observed in the reaction of PNPA with basic anilines and the "saturation" with respect to PNPA concentration represents a change in rate-determining step as the reaction with AABS becomes rate determining. Based on the maximum observed rates of acylation, PNPA is approximately 300 times more reactive than phenyl acetate.

The reactivity of bound anilines increases by almost 2 orders of magnitude with increasing basicity of the aniline, but it is difficult to establish a quantitative relationship of reactivity with basicity because of the scatter of the data and the change in rate-determining step that occurs with anilines with $pK$ values greater than 4 (Fig. 6). It has already been noted that the decrease in $K_m$ values with increasing basicity of uncharged anilines in the $p$-nitroacetanilide reactions is consistent with an increase in reactivity associated with the increase in basicity. If it is assumed that meta-substituted anilines do not bind optimally to the active site and that the $p$-acetyl group of $p$-aminoacetophenone is conjugated with the aniline ring and gives a less favorable geometry than ethyl $p$-aminoacetate, in which conjugation of the bridging ester oxygen atom with the carbonyl group may permit rotation of this group out of the plane of the aromatic ring, then a line of slope $\beta = 0.6$ may be drawn through the remaining weakly basic anilines, as shown in Fig. 6, to give an approximate measure of the sensitivity of the reaction rate to aniline basicity. These assumptions receive some support from the observation that the $K_m$ values for meta-substituted anilines are larger than those for other weakly basic anilines (Table III). The $K_m$ value for aniline itself is larger than the value for any of the substituted anilines, which suggests that substituents, especially in the para position, provide additional binding energy for attachment to the active site; the smaller values for the more basic anilines presumably also reflect the presence of the rate constant for the deacylation step in the observed $K_m$ value. Hansch, Deutsch, and Smith have pointed out previously that the reaction rate of a series of anilines, measured at a single concentration, is increased by hydrophobic substituents, presumably as a consequence of an increased binding to the enzyme (7, 31). It is probable that the relatively small differences in the rates of reaction of uncharged anilines in this series are a consequence of a kinetically significant rate of enzyme acylation with the more reactive anilines and of differences in aniline binding.

The dependence of the nonenzymatic reaction rate of substituted anilines with the ester 2,4-dinitrophenyl acetate upon the basicity of the aniline is large, with a $\beta$ value of 0.9 from the slope of a plot of log $k$ against $pK$ (22). This value is similar to $\beta$ values of 0.8 to 1.0 for the nonenzymatic reactions of a variety of amines of a given structural class with esters and thiol esters of moderate reactivity (23-25). A $\beta$ value of 0.9 means that the rate of the aminolysis reaction is 0.9 as sensitive to polar substituents as is the equilibrium constant for protonation; i.e., the reaction behaves as if some 0.9 positive charge has developed on the attacking nitrogen atom in the transition state. This and other evidence (32) indicates that in the transition state for the nonenzymatic reaction there is a large amount of bond formation to the attacking nitrogen atom that causes positive charge development and little or no proton removal from this atom, which would decrease the amount of positive charge development. This conclusion may be summarized by transition state I, in which no position is taken as to whether the rate-determining step involves the formation, the breakdown, or both, of a tetrahedral addition intermediate.

\[
\begin{align*}
\text{I} & \quad \text{H-N-C-O} \quad \text{(+) O (-)} \\
\text{PNPA} & \quad \text{B-H-N-C-O} \quad \text{(-) O (+)}
\end{align*}
\]

\[
\begin{align*}
\text{II} & \quad \text{H-N-C-O} \quad \text{(+) O (-)} \\
\text{PNPA} & \quad \text{B-H-N-C-O} \quad \text{(-) O (+)}
\end{align*}
\]

\[
\begin{align*}
\text{III} & \quad \text{B-H-N-C-O} \quad \text{(-) O (+)} \\
\text{PNPA} & \quad \text{B-H-N-C-O} \quad \text{(-) O (+)}
\end{align*}
\]

\[
\begin{align*}
\text{IV} & \quad \text{B-H-N-C-O} \quad \text{(-) O (+)} \\
\text{PNPA} & \quad \text{B-H-N-C-O} \quad \text{(-) O (+)}
\end{align*}
\]

The large sensitivity of the rate constant for the reaction of anilines with the acetyl-enzyme intermediate of acetyltransferase to the basicity of the aniline means that there is a large amount of charge development on the attacking nitrogen atom, as in the nonenzymatic reaction; the $\beta$ value of 0.6 may indicate a relatively small amount of proton removal by a catalyzing base that decreases the amount of charge development below that corresponding to the $\beta$ value of 0.9 for the nonenzymatic reaction, so that the transition state may be formulated as in II. This is in marked contrast to the low sensitivity to basicity observed in the reactions of anilines with furyl-chymotrypsin, which exhibits a $\beta$ value of 0.1 to 0.2 (38). The simplest interpretation of this low $\beta$ value is that the development of charge on the attacking nitrogen atom caused by bond formation is almost entirely balanced by charge removal resulting from the partial abstraction of a proton by a general base at the active site, presumably imidazole, so that the chymotrypsin reaction may be
formulated according to transition state III. The reactions of alcohols with furoyl-chymotrypsin display little or no sensitivity to basicity, as in the amine reactions, but the rates of the reactions of alcohols with acetyl-esterase show a considerable increase with increasing basicity, so that these reactions may be formulated according to transition states III and II, respectively, with oxygen replacing the attacking nitrogen atom (33, 34). Finally, the nonenzymatic reactions of alcohols with esters near neutrality proceed through the alkoxy anion with complete proton removal from the attacking oxygen, so that the observed reaction rates of the alcohols decrease with increasing basicity and may be formulated according to transition state IV (33).

For a reversible reaction (Equations 9 and 10), the sensitivity to polar substituents of the rate constant $k_r$ for the reaction in

$$Ac-E-RNH_2 \xrightarrow{k_f} AcNRH \cdot E$$

the reverse direction is determined by substituent effects on the rate constant in the forward direction and on the equilibrium constant according to the relationship $eta = \beta_f - \beta_{eq}$. Thus, the increase in rate with electron-donating substituents on alcohols attacking acetyl-esterase and the almost complete insensitivity to substituents of the rate of acetyl-esterase formation from esters are a consequence of the fact that the equilibrium constant (or acetyl transfer to substituted alcohols) has a large sensitivity to substituents, with a $\beta$ value of 0.7 (29, 34). The $\beta$ value for the equilibrium transfer of acetyl groups to substituted anilines catalyzed by acetyltransferase has been shown to be 0.61 (22), and a similar value has been found for the equilibrium formation of N-alkylacetamides. This large effect on the equilibrium is a consequence of the strongly electron-withdrawing nature of the acyl carbonyl group and electron donation by resonance to the carbonyl oxygen atom (V). Thus, substituent effects on the equilibrium constants and on the rate constants in the forward direction for the acetyltransferase reactions should approximately cancel, with $\beta$ values on the order of 0.6 for each, and it is predicted that there should be only a small sensitivity to substituents of the $V_{max}$ values for the reactions of bound acetanilides with the enzyme to form the acetyl-enzyme. This implies that in this direction there has been sufficient proton donation to the leaving aniline to overcome any diminution of positive charge caused by cleavage of the C-N bond, so that the partial positive charge on the nitrogen atom of the starting acetanilide (V) is maintained in the transition state (II). The available data are insufficient to permit a rigorous test of this prediction, but suggest that the substituent effects on the rate and equilibrium constants do largely cancel each other, leaving a relatively small accelerating effect of electron-withdrawing substituents in the acetyl-esterase on the formation of the acetyl-enzyme. Jacobson (7) has reported $V_{max}$ values for the reactions of substituted acetanilides with AABS that increase 3-fold as the basicity of the parent aniline decreases by a factor of $10^6$.

A. R. Fersht and Y. Requena, personal communication.

\[ \beta = \beta_f - \beta_{eq} \]

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**Fig. 8.** The effects of polar substituents, as estimated from the $\beta$ values for protonation, on the stabilities of amines, amides, and the transition states for several enzymatic and nonenzymatic reactions.

**Fig. 9.** The effects of polar substituents on the stabilities of alcohols, esters, and transition states, as in Fig. 8.
direction are made. A comparison may also be made of the rate constants for the second order reactions in the two directions based on $V_{\text{max}}/K_M$ values. There is a trend toward an increase in these values with increasing basicity of the aniline for the reaction in the forward direction (Table III) and an opposite trend for three of the four acetonilides examined by Jacobson in the reverse direction, but there is a large amount of scatter resulting from irregular effects of substituents on $K$ and $K_M$ values (Table III) and, again, no quantitative conclusions may be drawn from these data.

On the other hand, the maximal rate of acetyl-envelope formation from PNPA is some 200 times greater than that observed with phenyl acetate, so that it is apparent even from just these two compounds that there is a large sensitivity of the rate of acylation by esters to the basicity of the leaving group. This suggests that for these relatively good leaving groups, in contrast to the amine-amide reactions, proton donation to the leaving oxygen atom is of little or no importance. A similar difference between the behavior of ester and amide substrates has been observed for papain (35).

These considerations for acetyltransferase and other acyl group transferring enzymes may be summarized schematically as shown in Fig. 8 for amine-amide reactions and Fig. 9 for alcohol-ester reactions. In each case, the substituent sensitivities of the rate constants and the development of charge on the attacking or leaving atom are different in the two directions because of the rate constants and the difference in the charge of this atom in the starting materials and products. In the case of the acetyltransferase and liver esterase (34), the available measurements of reaction rates in the two directions are consistent with the equilibrium constant effects, but in the case of chymotrypsin there is a discrepancy between the conclusions reached from examination of the second order reactions of alicyclic amines with the acyl-envelope and the $V_{\text{max}}$ values for the reactions of acetytyrosine anilides (32, 36, 37), discussed in more detail elsewhere (22), that may be partially accounted for by the presence of polar substituents on substrate binding. There is a smaller difference between the substituent effects on the alcoholysis of fureolyl-chymotrypsin ($\beta = 0$) and on enzyme acylation by phenyl esters, for which the $\beta$ value for the leaving group is $-0.2$ relative to the starting ester (38), that presumably reflects a difference in the behavior of structurally different substrates. The important conclusion from Figs. 8 and 9 is that there is a wide range of substituent sensitivities for enzymatic acyl group transfer reactions that may be accounted for by a corresponding range in the extent to which charge development consequent upon $C-O$ or $C-N$ bond formation or cleavage is balanced by proton removal or addition. We believe that this spectrum of behavior is most easily interpreted in terms of variable amounts of general acid or base catalysis of these reactions by appropriate groups in the active site of the enzymes and, in fact, that this type of data provides the most direct evidence so far available supporting the existence of such catalysis. These data provide no support for mechanisms in which the proton is completely transferred before or after the transition state is reached (39) and, although it is difficult to evaluate the exact timing of the proton transfer process, we believe that it is conceptually more reasonable to expect most enzymatic reactions to proceed through the usual mechanism of "concerted" or "coupled" general acid-base catalysis rather than through an energetically unfavorable pre-equilibrium proton transfer step for which there is no obvious driving force or mechanistic advantage.

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