Free Energies of Acetyl Transfer from Ring-Substituted Acetanilides[1]

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Abstract: Equilibrium constants for transfer of the acetyl group from a series of aniline-substituted acetanilides to aminoazobenzenesulfonic acid, catalyzed by acetyl CoA–arylamine *N*-acetyltransferase, are reported. A logarithmic plot of the equilibrium constant for acetyl transfer against the basicity of the parent aniline has a slope $\beta = -0.61$, which indicates that the sensitivity to polar substituent effects of acetanilides is similar to that of other compounds in which oxygen or nitrogen, but not sulfur, is acylated. The better fit to σ^- than to σ substituent constants supports the conclusion that resonance interaction between the lone-pair electrons and the aromatic ring of aniline is lost upon acetylation. The rate constants for the reactions of substituted anilines with 2,4-dinitrophenyl acetate give a β value of 0.91. The structures of reactants and transition states are compared for some reactions of anilines and other amines with acyl compounds, with special reference to the discrepancy between structure–reactivity correlations for the reactions of furoyl-chymotrypsin with amines and of substituted anilides with chymotrypsin.

We report here a study of the effect of polar substituents in the aniline moiety on the stabilities of a series of acetanilides. A comparison of the effects of substituents on the overall equilibrium constants for acetanilide formation and on rate constants for reactions of anilides and anilines is useful in providing a more complete description of the structure and position of the transition state along the reaction coordinate for these reactions in both directions. Previously obtained data on the free energies of hydrolysis of esters and acetylpyridinium ions have permitted the use of this approach in reactions involving the formation or cleavage of these compounds.[2-4] The present work was carried out with substituted acetanilides to evaluate the electronic structure of these compounds in comparison with other acyl compounds and to permit an evaluation of chemical and enzymatic reaction mechanisms, with special reference to the acylation of anilines catalyzed by acetyl coenzyme A–arylamine *N*-acetyltransferase[5] and the reactions of chymotrypsin with anilides, anilines, and other amines. Equilibrium constants for the formation of substituted formanilides have been measured previously at 100° in 2:1 pyridine–water,[6] but it was desirable to obtain data for acetanilides at 25° in water for comparison with kinetic data.

Experimental Section

Materials. Anilines were redistilled under reduced pressure or recrystallized. Acetanilides were prepared as described by Fieser[7] and recrystallized. 4-Aminoazobenzene-4′-sulfonic acid (AABS) and its *N*-acetyl derivative (AcAABS) were kindly donated by Dr. S. P. Bessman. We have confirmed a previously reported value[8] of ϵ 1640 at 460 nm for AcAABS at pH 8.5, but find a value of ϵ 10,500 at 460 nm at pH 8.5 for AABS which is larger than a previously reported value,[8] probably because of a difference in pH. 2,4-Dinitrophenyl acetate was prepared by Dr. J. Kirsch. Other reagents were the best obtainable commercial grade. Partially purified arylamine transacetylase was prepared by Dr. B. Riddle.[5]

Determination of Equilibrium Constants. Equilibria for the transfer of acetyl groups between AABS and a series of substituted anilines were measured spectrophotometrically by following the change in absorption of free AABS at 460 and 440 nm; agreement within 5% (usually 2%) was obtained from the results at the two wavelengths. Experiments were carried out in stoppered cuvettes at pH 8.5, 25°, in the presence of 0.1 *M* tris(hydroxymethyl)-aminomethane hydrochloride buffer, 0.01 *M* ethylenediaminetetraacetic acid, and 0.001 *M* dithiothreitol. The solutions were deoxygenated by bubbling with argon for 5 min before the addition of enzyme. In each experiment the total concentration of AABS + AcAABS was 5×10^{-5} *M* and the ratio of acetylated to free dye at equilibrium was determined from eq 1, in which A_{eq} is the observed absorbance at equilibrium and A_N and A_{Ac} are the absorbancies

$$\frac{[\text{AcAABS}]}{[\text{AABS}]} = \frac{A_N - A_{eq}}{A_{eq} - A_{Ac}} \quad (1)$$

of 5×10^{-5} *M* free and acetylated dye, respectively. The concentrations of free and acetylated dye added were directly measured spectrophotometrically in each experiment. Each equilibrium constant was measured at least once in an experiment in which equilibrium was approached from both directions, starting with free and with acetylated dye, and from an approximately equimolar mixture of free and acetylated dye.

In the case of *p*-nitroacetanilide the equilibrium was measured for acyl transfer to *p*-aminoacetophenone, instead of AABS, by following the absorption of *p*-nitroaniline at 424 (ϵ 7850) and 414 nm (ϵ 5300).

Although certain preparations of the enzyme catalyze a slow hydrolysis of some acetanilides, there was no evidence for significant hydrolysis in the presence of added anilines in the equilibrium experiments as shown by the constancy of equilibrium values approached from both directions and of control readings in runs containing AcAABS or AcAABS and acetanilide, but no free aniline.

Determination of Rate Constants. Second-order rate constants for the reactions of anilines with 2,4-dinitrophenyl acetate were measured spectrophotometrically by following the release of 2,4-dinitrophenolate ion at 400 nm by the method of initial rates or by measurement of pseudo-first-order rate constants in the presence of a large excess of aniline. The experiments were carried out at an ionic strength of 1.0, maintained with potassium chloride, in a

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(2) J. Gerstein and W. P. Jencks, *J. Amer. Chem. Soc.*, **86**, 4655 (1964).

(3) W. P. Jencks and M. Gilchrist, *ibid.*, **90**, 2622 (1968).

(4) A. R. Fersht and W. P. Jencks, *ibid.*, **92**, 5432, 5442 (1970).

(5) B. Riddle and W. P. Jencks, *J. Biol. Chem.*, **246**, 3250 (1971).

(6) O. C. M. Davis, *Z. Phys. Chem.*, **78**, 353 (1912); O. C. M. Davis and F. W. Rixon, *J. Chem. Soc.*, 107, 728 (1915).

(7) L. Fieser, "Experiments in Organic Chemistry," 3rd ed, D. C. Heath, Boston, Mass., 1957, pp 151–152.

(8) K. B. Jacobson, *J. Biol. Chem.*, **236**, 343 (1961).

Table I. Equilibrium and Rate Constants for the Acetylation of a Series of Substituted Anilines at 25°

Aniline substituent	K_{eq}[a]	pK_a[b]	σ[c]	σ^-[d]	[AcNHR],[e] $M \times 10^3$	[RNH_2],[e] $M \times 10^4$	k_2,[f] M^{-1} min^{-1}	[RNH_2], $M \times 10^3$	$\Delta G_{hyd}°$,[g] kcal/mol
p-CH_3O–	0.017	5.34	−0.27		3.0	0.5	75	3.0–12	3.7
p-CH_3–	0.034	5.07	−0.17		3.0	1.0	37	1.5–5.0	3.2
H–	0.06	4.60	0		6.0	4.0	13.2	25–100	2.8
p-Cl–	0.13	3.98	0.23		0.5	0.7	4.6	3.0–10	2.4
m-CF_3–	0.23	3.49	0.43		1.5	4.0	1.24	1.6–6.0	3.0
p-C_2H_5OOC–	0.94	2.51	0.45	0.678	0.6	6.0	0.22	0.8–3.0	1.2
p-CH_3CO–	1.7	2.19	0.50	0.874	0.6	10			0.8
p-CN–	3.7	1.74	0.66	1.00	0.3	10			0.4
p-NO_2–	8.5[h]	1.01	0.78	1.27	0.1	1.0			−0.1

[a] K_{eq} = [AcAABS][RNH_2]/[AABS][AcNHR]. [b] In water at ionic strength <0.02 (A. I. Biggs and R. A. Robinson, *J. Chem. Soc.*, 388 (1961); J. D. Roberts, R. L. Webb, and E. A. McElhill, *J. Amer. Chem. Soc.*, **72**, 408 (1950); J. M. Vandenbelt, C. Henrich, and S. G. Vanden Berg, *Anal. Chem.*, **26**, 726 (1954); M. M. Fickling, A. Fischer, B. R. Mann, J. Packer, and J. Vaughan, *J. Amer. Chem. Soc.*, **81**, 4226 (1959)). [c] D. H. McDaniel and H. C. Brown, *J. Org. Chem.*, **23**, 420 (1958). [d] See Biggs and Robinson, footnote *b*; H. H. Jaffé, *Chem. Rev.*, **53**, 191 (1953). [e] Approximate final concentration. [f] In 0.7% acetonitrile; ionic strength maintained at 1.0 with potassium chloride. [g] Approximate value, based on $\Delta G_{hyd}°$ for formanilide (see text). [h] Obtained by equilibrating *p*-nitroaniline (free and acetylated) with *p*-aminoacetophenone (free and acetylated) and comparing with the equilibrium constant for the reaction of *p*-aminoacetophenone with AcAABS.

buffer of the aniline and its hydrochloride, except for the reaction with ethyl *p*-aminobenzoate which was carried out in 0.2 *M* acetate buffer, pH 4.78. Solutions of 2,4-dinitrophenyl acetate were prepared in acetonitrile and diluted with 4 vol of water before addition to reaction mixtures to give a final concentration of 3×10^{-5} *M* (pseudo-first-order) or 3×10^{-4} *M* (initial rate experiments) and a final concentration of acetonitrile of 0.7%. End points for the initial rate experiments were obtained in the appropriate buffer using an aliquot of ester that had been subjected to alkaline hydrolysis (1 hr, 0.01 *M* KOH) and neutralized. Second-order rate constants were obtained from the slope of a plot of the observed first-order rate constants against the concentration of free aniline. First-order rate constants were obtained from the slope of the initial rate or from the half-time for *p*-nitrophenolate appearance.

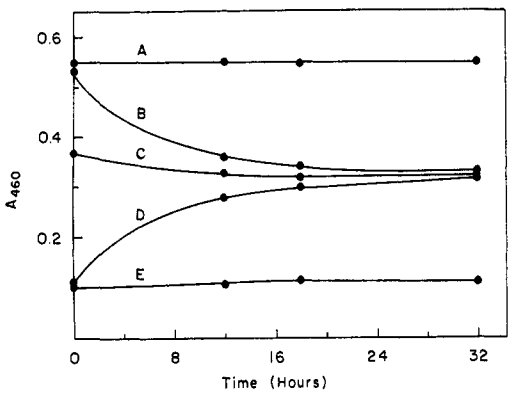


Figure 1. Approach to equilibrium for acetyl transfer between AABS and *p*-chloroaniline catalyzed by acetyltransferase (30 μg/ml) at pH 8.5, 25°. See Table I and text for experimental conditions: A, AABS and *p*-chloroaniline; B, C, and D, experimental runs; E, AcAABS and *p*-chloroacetanilide.

All of the anilines except for ethyl *p*-aminobenzoate were measured by the method of pseudo-first-order rate constants; the rate constants for this compound and for *m*-trifluoro- and *p*-chloroaniline were determined by the method of initial rates. Four to eight rate determinations were carried out for each aniline. With ethyl *p*-aminobenzoate, the least reactive aniline examined, the observed rate constants increased from 0.8 to 1.4×10^{-3} min^{-1} with increasing aniline concentration.

The correlation lines of Figures 2–4 were calculated by the method of least squares.

Results

The free energies of hydrolysis of substituted acetanilides (eq 2) were measured relative to that of *N*-acetylaminoazobenzenesulfonic acid (AcAABS, eq 3) by determining the equilibrium constant for acetyl transfer between acetanilides and aminoazobenzenesulfonic acid (AABS, eq 4). This dye has an absorption in the visible region and serves as a convenient indicator of the "acetyl potential" of a solution containing acetanilide and free aniline in the same way that a pH indicator serves for the determination of hydrogen ion activity. Equilibrium was approached from both directions and also measured in a run containing a mixture of free and acetylated dye, as shown for the reaction of free and acetylated *p*-chloroaniline in Figure 1. The equilibrium constants obtained in this manner for the series of compounds examined are summarized in Table I, along with the concentrations of aniline and acetanilide in the experimental runs. The values are estimated to be accurate to within 10%.

$$\text{AcNHArX} + \text{H}_2\text{O} \rightleftharpoons \text{AcOH} + \text{H}_2\text{NArX} \quad (2)$$

$$\text{AcAABS} + \text{H}_2\text{O} \rightleftharpoons \text{AcOH} + \text{AABS} \quad (3)$$

$$\text{AcNHArX} + \text{AABS} \rightleftharpoons \text{AcAABS} + \text{H}_2\text{NArX} \quad (4)$$

Second-order rate constants for the nonenzymatic reactions of 2,4-dinitrophenyl acetate with the more basic anilines, which could be measured without serious interference from hydrolysis, are also summarized in Table I.

Discussion

The equilibrium constants for the transfer of an acetyl group from a series of substituted acetanilides to a constant acyl acceptor (Table I) show that the free energy of acetanilides relative to the corresponding aniline is increased by electron-withdrawing substituents in the aniline; the equilibrium constant for acetyl donation from *p*-nitroacetanilide is 500-fold larger than that for the reaction of *p*-methoxyacetanilide, for example. The absolute value for the free energy of hydrolysis of acetanilide is not known, but an approximate value may be obtained from the free energy of hydrolysis of formanilide,[9,10] in view of the fact that the equilibrium constants for the hydrolysis of the *N*-acetyl and *N*-formyl derivatives of thiosemicarbazide do not differ significantly.[10] The equilibrium constant for

(9) I. Öney and M. Caplow, *J. Amer. Chem. Soc.*, **89**, 6972 (1967).
(10) A. R. Fersht and Y. Requena, personal communication.

formanilide hydrolysis has been found[9,10] to be 0.046 at 50° and 0.0085 at 25°, giving a *positive* free energy of hydrolysis of 2.82 kcal/mol at 25° (based on the uncharged species of the reactants and an activity of pure water of 1.0). Based on the formanilide value, the absolute values for the free energies of hydrolysis of substituted acetanilides to uncharged products range from 3.7 kcal/mol for *p*-methoxyacetanilide to −0.1 kcal/mol for *p*-nitroacetanilide (Table I); at pH 7.0 the free energies are more negative by the free energy of ionization of acetic acid of −3.3 kcal/mol (at pH 7.0, based on $pK' = 4.61$ at ionic strength 1.0)[3] and range from 0.4 to −3.2 kcal/mol.

A logarithmic plot of the equilibrium constants for acetyl transfer from substituted acetanilides against the pK_a values of the parent anilines is linear with a slope of $\beta = -0.61$ (Figure 2). The β value of 0.61 in the reverse direction, *i.e.*, for *replacing* a proton by an acetyl group in a series of substituted anilines, may be compared to the β value of 1.0 for adding a proton and means that the free-energy change associated with the development of a partial positive charge on the aniline nitrogen atom upon acetylation is 0.6 as large as the free-energy change associated with the development of a full positive charge upon protonation. This value is similar to the β value of 0.70 for *replacing* the proton of an alcohol by an acetyl group[2] and may be compared to the values of 1.70 and 1.60 for the *addition* of an acetyl group to a substituted alcoholate anion or pyridine,[4] respectively; in the former case the values of 0.7 and 1.7 differ by the β value of 1.0 for ionization of the alcohol. The β value of 0.61 is close to that of 0.51 obtained from the data of Davis for the formation of substituted formanilides at 100° in 2:1 pyridine–water[6] and is also similar to the value of 0.51 for the formation of a series of *N*-alkylacetamides found by Fersht and Requena[10] in an investigation complementary to that reported here.

It should be noted that the β values for acetylation of alcohols and amines are not strictly comparable, because the ionization constants refer to the loss of a proton from the alcohol itself in the former case and to the loss of a proton from the conjugate acid of the amine in the latter case. Nevertheless, the β values serve as a useful measure of the sensitivity of these compounds to charge development on the atom that is acetylated and exhibit an extraordinary constancy at 0.6 ± 0.1 for the addition of an acetyl group to nitrogen or oxygen over a large range of structural variation. These values confirm the strongly electrophilic character of the acyl group, which is more electron withdrawing than the proton,[2,11] and may be loosely interpreted in terms of the development of some 0.6 positive charge on the nitrogen or oxygen atom upon acetylation. The striking similarity of this value for esters, amides, acetanilides, and *N*-acetylpyridinium ions, in spite of the large differences in the structure and degree of resonance stabilization of these compounds, suggests that there may be a compensatory phenomenon whereby a decrease in positive charge development by resonance is counteracted by an increased sensitivity to the inductive electron-withdrawing power of the acyl group in going from the strongly resonance-stabilized amides through

(11) J. Hine and R. D. Weimar, Jr., *J. Amer. Chem. Soc.*, 87, 3387 (1965).

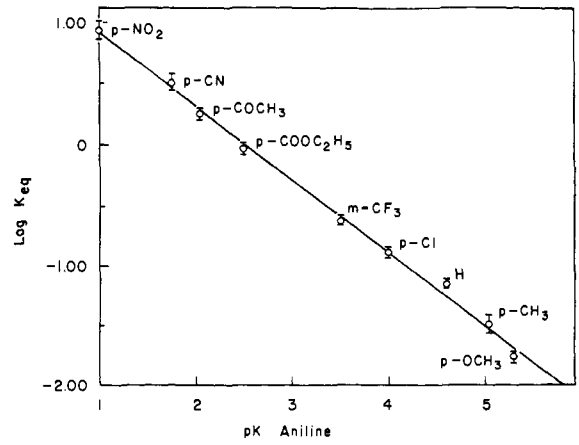
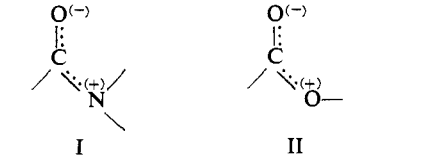


Figure 2. Log K_{eq} for acetyl transfer from substituted acetanilides to AABS at 25° as a function of the pK of the aniline; slope = −0.61. The bars show the range of observed values.

esters to acetylpyridinium ions (I–III). That this β value and compensation are not completely general is shown by the almost complete insensitivity to the acidity of the parent thiol of the free energies for acetyl transfer from a limited series of thiol esters.[12] This implies that there is little or no change in the charge on the sulfur atom in going from the free thiol to the thiol ester. This large difference between the electron distribution of thiol esters and of other acyl compounds provides strong evidence in support of the conclusion that in thiol esters positive charge development on sulfur caused by electron withdrawal to the carbonyl group is effectively cancelled by negative charge development by electron donation from the carbonyl group into a sulfur d orbital, IV,[13] a conclusion that has recently been questioned on the basis of an evaluation of infrared absorption spectra.[14]



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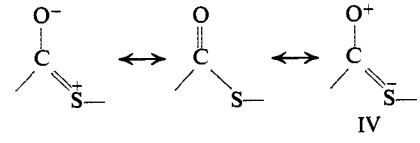


IV

A plot of log K for the acetanilide reactions against σ and σ^-, shown in Figure 3, gives a value of $\rho^- = 1.73$. The much better fit to the σ^- parameters, which take account of electron withdrawal by resonance, compared to σ and the fit to the correlation with basicity (Figure 2) provide direct evidence supporting the generally held view[15] that the resonance stabilization involving the lone-pair nitrogen electrons of free aniline is almost completely lost upon acetylation, as upon protonation, because of the overriding demand for this

(12) J. J. O'Neill, H. Kohl, and J. Epstein, *Biochem. Pharmacol.*, 8, 399 (1961).

(13) A. W. Baker and G. H. Harris, *J. Amer. Chem. Soc.*, 82, 1923 (1960); I. Wallmark, M. H. Krackov, S-H Chu, and H. G. Mautner, *ibid.*, 92, 4447 (1970).

(14) A. J. Collings, P. F. Jackson, and K. J. Morgan, *J. Chem. Soc., B*, 581 (1970).

(15) R. E. Carter, *Acta Chem. Scand.*, 21, 75 (1967), and references therein.

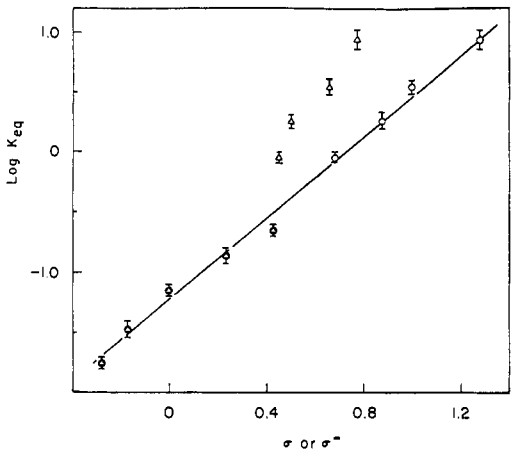
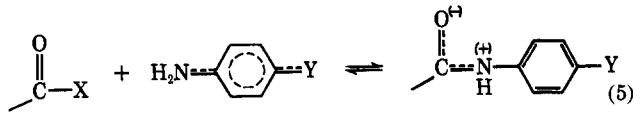


Figure 3. Hammett plots for K_{eq} for acetyl transfer from substituted acetanilides to AABS: O, σ^-; Δ, σ.

electron pair by the carbonyl group (eq 5). This is in



(5)

accord with the much larger estimated resonance stabilization energy of the amide group than of aniline[15] and suggests that even if resonance interaction between nitrogen and the aromatic ring exists in certain anilides,[16] it does not make an appreciable contribution to the free energy of acetanilides (or of formanilides, since the data of Davis[6] also give a good correlation with aniline basicity). The fact that formanilide is 1.4 kcal less stable than predicted by a correlation of amide stability with the basicity of aliphatic amines[10] is *not* a consequence of this resonance stabilization, because the stabilization is lost upon both acetylation and the reference protonation reaction; it may reflect a larger steric hindrance to the formation of anilides compared to aliphatic amides.

Structure–Reactivity Correlations. Knowledge of the effect of substituents on the equilibrium permits a more complete description of the charge distribution in the transition states of reactions involving the formation and cleavage of acetanilides. The second-order alkaline hydrolysis of acetanilides exhibits essentially no effect of substituents on reaction rate and it may be concluded from the results of ^{18}O exchange and kinetic studies with other anilides that the transition state for this reaction involves the breakdown of a tetrahedral addition intermediate with a net charge of -1.[17,18] The substituent effects on the rate and equilibrium constants for hydrolysis mean that the reaction behaves as if the aniline nitrogen atom has approximately the same charge in the transition state as in the starting acetanilide and some 0.6 more positive charge than in the product aniline (eq 6). This supports the earlier conclusion[17] that protonation of the leaving aniline (presumably by the solvated proton acting as a general acid or water acting as a cyclic proton transferring agent) is

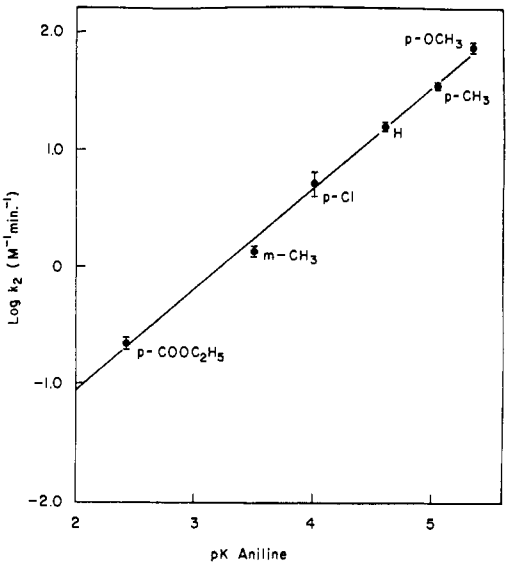
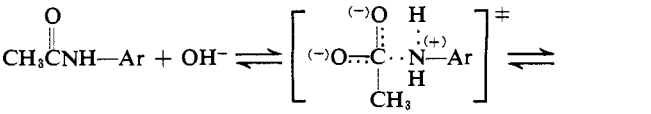


Figure 4. Second-order rate constants for the reactions of substituted anilines with 2,4-dinitrophenyl acetate at 25° as a function of aniline basicity; slope = 0.91. The bars show the range of observed rate constants.

important and that the balance between protonation and carbon–nitrogen bond breaking is such that a large net positive charge remains on the nitrogen atom in the transition state. The reverse reaction is the attack of

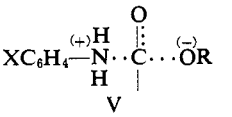


$CH_3COO^- + H_2NAr$ (6)

aniline on the acetate ion (possibly catalyzed by water acting as a general base) and the net development of some 0.6 positive charge on the attacking aniline means that carbon–nitrogen bond formation has proceeded to a greater extent than proton removal in the transition state; in fact this β value is very similar to the value of 0.7 for the attack of amines on methyl formate.[19] This degree of charge development is also consistent with the large amount of proton transfer to the leaving aniline that is indicated by the α value of 0.9 for general acid catalysis of the alkaline hydrolysis of trifluoroacetanilide.[20]

The β value of 0.9 for the reaction of substituted anilines with 2,4-dinitrophenyl acetate (Figure 4) is in agreement with the β value of 0.9 ± 0.1 for the reactions of primary, secondary, and tertiary amines with several substituted phenyl esters.[3,21] This β value, which is greater than that for the overall equilibrium, means that the large amount of positive charge development resulting from a large amount of nitrogen–carbon bond formation is not offset by a significant amount of proton removal from the attacking nitrogen atom in the transition state V. The β value for the reverse reaction, based on the rate constant for the reaction of free dinitrophenol with the substituted anilide, is 0.9 − 0.6 = 0.3, in agreement with the range of 0.3–0.5 cal-

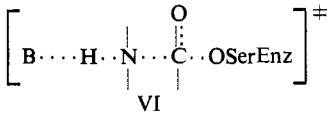
(16) P. Hampson and A. Mathias, *Mol. Phys.*, 11, 541 (1966); J. Niwa, *Bull. Chem. Soc. Jap.*, 42, 1926 (1969).
(17) M. L. Bender and R. J. Thomas, *J. Amer. Chem. Soc.*, 83, 4183 (1961).
(18) P. M. Mader, *ibid.*, 87, 3191 (1965); R. L. Schowen, H. Jayaraman, and L. Kerschner, *ibid.*, 88, 3373 (1966); S. O. Eriksson and C. Holst, *Acta Chem. Scand.*, 20, 1892 (1966).
(19) G. M. Blackburn and W. P. Jencks, *J. Amer. Chem. Soc.*, 90, 2638 (1968).
(20) S. O. Ericksson, *Acta Chem. Scand.*, 22, 892 (1968).
(21) T. C. Bruice and R. Lapinski, *J. Amer. Chem. Soc.*, 80, 2265 (1958).



V

culated for the reactions of phenols with a series of aliphatic amides.[10] This increase in rate with electron-*donating* substituents in the leaving group means that protonation of the leaving nitrogen atom is of overriding importance, so that there is an appreciable positive charge development on this atom in the transition state even compared to the starting anilide.

There is a striking contrast between these results and those for reversible reactions of acyl–serine ester intermediates of chymotrypsin with amines. The β value of 0.13–0.19 for the attack of aliphatic amines of pK 5.7–11.2 on the acyl–enzyme intermediate, furoylchymotrypsin, shows that there is very little development of positive charge on the attacking nitrogen atom in the transition state for this aminolysis reaction. This has been interpreted as evidence that the development of positive charge on the attacking amine in the transition state is effectively prevented by a compensatory removal of positive charge caused by a general base catalyzed proton abstraction[22] (VI; in this and the



VI

following transition states no position will be taken as to whether formation or breakdown of a tetrahedral addition intermediate is rate determining).[23] The reverse reaction, the rate-determining formation of an acyl–enzyme from an amide, has been studied with a series of aniline-substituted *N*-acetyltyrosine anilides and it has been found that, with the exception of the *p*-nitroanilide, the rate of formation of the acyl–enzyme from the bound substrate is markedly increased by electron-donating substituents;[24,25] the β value from a plot of log V_{max} against the pK of the aniline is 0.6. This means that there is a large development of positive charge on the nitrogen atom in going from the bound anilide to the transition state of this reaction and is most easily interpreted as evidence for a large amount of proton transfer to this nitrogen atom.[24–26] This is consistent with the conclusion that there is proton removal from the amine in the deacylation reaction, the microscopic reverse of acylation. However, there is a large quantitative discrepancy between the β values for the reactions in the two directions that becomes even more serious when account is taken of the β value for

(22) There are two limiting, but less probable, cases of this interpretation: (1) The forward reaction involves rate-determining breakdown of a tetrahedral addition intermediate from which the amine proton has been completely removed; in the reverse direction this transition state corresponds to partial formation of the carbon–oxygen bond that causes an increase in electron density on the amide nitrogen atom, with no proton donation to this atom. (2) The transition state occurs very early along the reaction coordinate with little N–C bond formation, as in the reactions of basic amines with highly reactive acylating reagents;[3] in the reverse reaction this would correspond to a late transition state with essentially complete protonation of the leaving amine.

(23) P. W. Inward and W. P. Jencks, *J. Biol. Chem.*, 240, 1986 (1965).

(24) T. Inagami, S. S. York, and A. Patchornik, *J. Amer. Chem. Soc.*, 87, 126 (1965); T. Inagami, A. Patchornik, and S. S. York, *J. Biochem. (Tokyo)*, 65, 809 (1969).

(25) L. Parker and J. H. Wang, *J. Biol. Chem.*, 243, 3729 (1968).

(26) W. F. Sager and P. C. Parks, *J. Amer. Chem. Soc.*, 85, 2678 (1963).

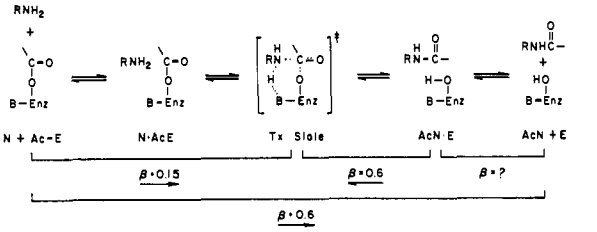


Figure 5. Effect of amine basicity, expressed as β values, on the steps of the reaction of acylchymotrypsin with amines and the reverse reaction.

the overall equilibrium (Figure 5). If, for purposes of discussion, one considers the β values as a direct measure of the amount of charge development on the amine nitrogen atom, then there is less than +0.2 positive charge development in going from the free aniline to the transition state, there is 0.6 charge development in going from the aniline to the acetanilide, and there is an additional positive charge development of 0.6 in going from the bound anilide to the transition state. If it is assumed that there is no effect of polar substituents on the binding of anilide, then the difference of positive charge between free aniline and the transition state for the anilide reaction is +1.2, in marked disagreement with the previously noted value of <0.2. There is even more positive charge development than in the acid-catalyzed hydrolysis of acetanilides, which exhibits a small *decrease* in rate with electron-donating substituents.[27]

If it is assumed that there are no serious errors in the experimental data, there are two possible explanations for this discrepancy. The data suggest that both contribute to the observed differences in substituent effects in the forward and reverse reactions.

(1) The reactions of aliphatic amines and amides involving the furoyl group may proceed through a transition state that is different from that for the reactions of anilines and anilides involving the acetyltyrosine group. It does not seem probable that a large difference of this kind is simply a consequence of the difference in amine basicity in the two reaction series because there is no evidence of developing curvature in the Brønsted plot for the aliphatic amines of pK as low as 5.7 nor in the aniline series for acylated anilines of pK as high as 5.3. There is no indication in the amine series that the β value changes with the structure of the amine, but the possibility remains that the aromatic ring of the anilines or the differences in the structure of the acyl group bring about a different geometry and charge distribution in the active site.

(2) The difference may be, in part, a consequence of the fact that the amine series involves a second-order reaction of free amine in solution, whereas the anilide reaction involves the bound substrate; *i.e.* the difference could reflect an effect of substituents on substrate binding. It is difficult to evaluate this possibility rigorously because of specific, presumably steric, effects on the individual K_m values, but there is a definite increase in K_m values with electron-withdrawing substituents which tends to counteract the effect on V_{max}, so that if the anilide reactivities are expressed as

(27) D. D. Karve and B. W. Kelkar, *Proc. Ind. Acad. Sci., Sect. A*, 24, 254 (1946); V. F. Mandyuk and N. P. Lushina, *Ukr. Khim. Zh.*, 32, 607 (1966) (*Chem. Abstr.*, 65, 15175 (1966)); E. Y. Belayev, L. I. Kotlar, G. L. Lysenko, and G. V. Pestova, *Org. React.*, 6, 980 (1969).

second-order rate constants for reaction from free solution, V_{max}/K_m, there is essentially no effect of substituents on the rate.[24] The increase in binding with electron-withdrawing substituents that is required by this hypothesis implies that the binding step brings the substrate part way along the reaction coordinate toward the transition state with respect to the chemical reaction itself, as well as with respect to the entropy of approximation of reacting groups. The limiting case of this situation would be the utilization of the substrate binding energy to force the formation of a tetrahedral addition intermediate as a part of the binding step.[28]

(28) M. Caplow, *J. Amer. Chem. Soc.*, 91, 3639 (1969).

The substituent effect on the overall equilibrium for acetanilide formation also provides an explanation for the fact that electron-withdrawing substituents decrease the rate of reaction of anilines with the acetyl–enzyme intermediates formed from acetyl-coenzyme A–arylamine acetyltransferase, but increase the rate of formation of this intermediate from substituted acetanilides; this reaction will be described in greater detail elsewhere.[5]

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Studies on the Carbon-13 Contact Shifts of a σ-Electron System. Conformational Dependence of Carbon-13 Contact Shifts in Six-Membered Rings[1]

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Abstract: Isotropic ^{13}C paramagnetic shifts for piperidine derivatives I–IV, quinuclidine (V), and 1-azaadamantane (VI) coordinated with paramagnetic nickel(II) acetylacetonate ($Ni(AA)_2$) have been observed in the completely proton-decoupled ^{13}C nmr spectra. These shifts with alternation in sign and attenuation in magnitude have been interpreted as due to Fermi contact shifts which are related to the electron spin densities on the carbon s atomic orbital. Different features of ^{13}C contact shifts were observed for N–H piperidines and *N*-methylpiperidines. For *N*-methylpiperidines, in which the lone-pair electrons are situated preferably at the axial position, α, β, and γ carbons experience alternate upfield and downfield ^{13}C contact shifts with rapid attenuation, while for N–H piperidines or 1-azaadamantane, in which lone-pair electrons have greater preference for the equatorial position, alternate ^{13}C contact shifts with slow attenuation were observed. For quinuclidine, however, an unexpected downfield ^{13}C contact shift (upfield shift for piperidine derivatives) was observed for the γ-carbon. These conformational dependencies of ^{13}C contact shifts have been discussed in terms of electron spin densities on the carbon atoms obtained by unrestricted Hartree–Fock SCF–MO calculations (INDO method). The experimental trends were reproduced by MO theoretical calculations for a cation radical in which an electron is abstracted from the neutral ligand molecule.

In this laboratory we have been interested in the nmr contact shifts for σ-electron systems and in their use in the elucidation of the mode of electron spin distribution through the σ skeleton and in the determination of molecular conformation. We have previously reported the proton contact shifts for piperidine,[3,4] quinuclidine,[3,4] aziridine,[5] ketoxime,[6] and aniline,[7] all of which have the oriented nitrogen lone-pair electrons serving as the σ binding site with the paramagnetic nickel(II) acetylacetonate ($Ni(AA)_2$). The proton contact shifts induced by the paramagnetic complexation have been shown to depend on the orientation of the lone-pair electrons and to be useful as a probe for studying molecular conformations.

Availability of the proton-decoupled ^{13}C nmr technique enables us to observe natural abundance ^{13}C nmr spectra of paramagnetic molecules in solution.[8] Recently we have reported[9,10] the ^{13}C nmr contact shift studies for aniline[9] and pyridine[10] complexed with

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(1) For preliminary research of this work, see I. Morishima, K. Okada, T. Yonezawa, and K. Goto, *Chem. Commun.*, 1535 (1970).

(2) Japan Electron Optics Laboratory Co., Ltd., Akishima, Tokyo, Japan.

(3) T. Yonezawa, I. Morishima, and Y. Ohmori, *J. Amer. Chem. Soc.*, 92, 1267 (1970).

(4) I. Morishima, K. Okada, M. Ohashi, and T. Yonezawa, *Chem. Commun.*, 33 (1971); *J. Amer. Chem. Soc.*, in press. The $Ni(AA)_2$-induced ^{13}C contact shifts give no information about the conformational equilibrium in piperidine itself. However, $Ni(AA)_2$ may not affect the lone-pair orientation, which is evidenced from the linear dependence of ^{13}C contact shift on the concentration of added $Ni(AA)_2$. If the conformational equilibrium is changed by the presence of $Ni(AA)_2$, the contact shift–concentration plots deviate from the linearity. This is also supported by the proton contact shift studies.[3]

(5) I. Morishima, K. Takeuchi, K. Fukuta, and T. Yonezawa, to be published.

(6) I. Morishima and T. Yonezawa, *J. Chem. Phys.*, 54, 3238 (1971).

(7) T. Yonezawa, I. Morishima, Y. Akana, and K. Fukuta, *Bull. Chem. Soc. Jap.*, 43, 379 (1970).

(8) D. Doddrell and J. D. Roberts, *J. Amer. Chem. Soc.*, 92, 4484, 5255, 6839 (1970).

(9) I. Morishima, T. Yonezawa, and K. Goto, *ibid.*, 92, 6651 (1970).

(10) I. Morishima and T. Yonezawa, submitted for publication.