ilar pK_a. It is also shown in the following paper[27] that the initial attack of methoxide ion on formylhydrazine to give the tetrahedral intermediate has a rate constant expected for that of the attack on an amide of a non-α-effect amine of similar pK_a.

The present ideas on the nature of the α effect are discussed in ref 10 and 28. The combination of our study and that of Blackburn and Jencks[5] requires, at least for the case of hydrazine and probably by analogy for hydroxylamine, that any explanation of the α effect based on either the increased stability of the resultant acylhydrazine or decreased stability of the nucleophile is highly suspect. The α effect for hydrazinolysis appears to be due to the increased stability of the transition state for tetrahedral intermediate formation.

The decreased thermodynamic stability of formamide deserves some comment. The geometries of NH_3 and NH_4^+ are highly compatible with that of water which could lead to high solvation energies,[29] but in formamide these interactions are lost. The nucleophilic reactivity of ammonia is not decreased with respect to primary and secondary amines by a factor equivalent to the decreased stability of the resultant amide so that any special solvation effects are not manifested in the transition states of ammonolysis reactions. The obvious structural differences of anilides and hydroxamic acids in relation to the other amides perhaps preclude direct comparison of their free energies of hydrolysis due to possible bond energy changes, but the similarity of formamide makes a solvation energy explanation attractive.

Acknowledgment. We wish to acknowledge a regular and stimulating exchange of information with Professor W. P. Jencks and permission to quote unpublished data.

(27) A. R. Fersht, *J. Amer. Chem. Soc.*, **93**, 3504 (1971).
(28) J. D. Aubort and R. F. Hudson, *Chem. Commun.*, 937, 938 (1970).
(29) See ref 21b, p 16.

Acyl-Transfer Reactions of Amides and Esters with Alcohols and Thiols. A Reference System for the Serine and Cysteine Proteinases. Concerning the N Protonation of Amides and Amide–Imidate Equilibria

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Abstract: The absolute magnitudes of the rate constants for the alcoholysis and thiolysis of some amides and esters have been obtained. Amides show the reactivity order: acylhydroxylamines $\gg$ acylhydrazines $\sim$ acyl primary amines $>$ acyl secondary amines. Within a single class of amides the reactivity increases with increasing pK_a of the amine moiety. Reactivity also increases with decreasing pK_a of the nucleophilic alcohol or thiol. In contrast, the reaction of alcohols with esters increases with increasing nucleophile pK_a and decreasing leaving group pK_a. The requirement for concurrent acid catalysis can lead to an alkoxide ion having a lower nucleophilicity than an apparently un-ionized alcohol. A method is given for the calculation of pK_a's for N protonation of amides. Comparisons are drawn between the enzymatic and nonenzymatic reactions and some speculated mechanisms are shown to be inconsistent. The resonance energy of amides is calculated to be 17–18 kcal/mol.

Despite the large amount of work done on the hydrolysis of peptides and amides by chymotrypsin and papain there are no systematic studies of the intermolecular alcoholysis of amides with which to provide a reference framework for the enzymatic studies. Structure–reactivity relationships have provided in past studies probably the most generally informative knowledge about the nature of chemical reactions. Some such studies have been performed with chymotrypsin, but without controlled chemical studies for comparison.

The absence of information on the alcoholysis of amides is due to the excessively slow rates of the uncatalyzed reactions. In a recent paper[1] we have emphasized the use of free energies of hydrolysis to obtain rate constants for experimentally inaccessible acyl-transfer reactions. Fortunately, the reverse reaction of amide alcoholysis, that is, ester aminolysis, is well studied.[2-5] The rate constants for this, in conjunction with the free energies of hydrolysis of amides which we have just measured,[6] and of esters, measured by Jencks and coworkers,[7-9] provide the rate constants for the alcoholysis reactions. The same is true for the thiolysis reactions. Combined with the fine studies from the laboratories of Schmir[10-13] and Jencks[14] on intramolecular

(1) A. R. Fersht and W. P. Jencks, *J. Amer. Chem. Soc.*, 92, 5442 (1970).
(2) W. P. Jencks and J. Carriuolo, *ibid.*, **82**, 675 (1960).
(3) G. M. Blackburn and W. P. Jencks, *ibid.*, **90**, 2638 (1968).
(4) T. C. Bruice, A. Donzel, R. W. Huffman, and A. R. Butler, *ibid.*, **89**, 2106 (1967).
(5) W. B. Jencks and M. Gilchrist, *ibid.*, **90**, 2622 (1968).
(6) A. R. Fersht and Y. Requena, *ibid.*, **93**, 3499 (1971).
(7) W. P. Jencks and M. Gilchrist, *ibid.*, **86**, 4651 (1964).
(8) J. Gerstein and W. P. Jencks, *ibid.*, **86**, 4655 (1964).
(9) W. P. Jencks, F. Barley, R. Barnett, and M. Gilchrist, *ibid.*, **86**, 4464 (1966).
(10) G. L. Schmir and B. A. Cunningham, *ibid.*, **87**, 5692 (1965).

alcoholysis and thiolysis and the paper by Blackburn and Jencks,[3] a picture of the intramolecular reactions is possible. It is hoped that the description of the reactions so obtained, and a discussion of the limitations in their characterization, will provide a rational basis for the description of some parts of the enzymatic mechanism.

Experimental Section

The free energy of hydrolysis of methyl formate was measured by the method of Jencks and Gilchrist:[7] 1 *M* methanol, 0.16 *M* formic acid, and various concentrations of methyl formate in 1 *N* HCl were allowed to equilibrate ($t_{1/2}$ = 6 min) at 25° and the concentration of methyl formate was assayed by the alkaline hydroxamate assay. The equilibrium constant, written as $[HCO_2Me]/([HCO_2H][MeOH])$, with a water activity of 1.0, is 0.122 ± 0.003, which may be compared with the value of 0.135 obtained under similar conditions for the hydrolysis of *N*-acetylphenylalanine methyl ester,[15] and also close to that predicted for methyl acetate.[7]

Method of Calculation

The equilibrium constant for

$$>NH + RCO_2R' \rightleftharpoons >N-\overset{\overset{O}{\|}}{C}-R + R'OH \qquad (1)$$

may be calculated simply from the free energies of hydrolysis written thus

$$>N-\overset{\overset{O}{\|}}{C}-R + H_2O \rightleftharpoons >NH + RCO_2H \qquad (2)$$

$$RCO_2R' + H_2O \rightleftharpoons RCO_2H + R'OH \qquad (3)$$

As far as possible, data collected at 25° and ionic strength 1.0 are used. However, equilibria such as 1, 2, and 3 should be somewhat insensitive to changes in ionic strength as no ions are involved. The use of such data at 30° should also lead to just a small error in the equilibrium constants. This last error, though not negligible, is insignificant in comparison with the knowledge obtained about the overall magnitude of the rate constants.

In the case of the equilibria with methyl formate and formamides the data used are precise. However, for acetates, the free energies for the amide hydrolysis are extrapolated from the formamide results as described in the accompanying paper. The errors involved in this are again insignificant in the context of use. The free energies of hydrolysis of some thiol esters have been determined[7,16] and, written as eq 2, are independent of the pK_a of the thiol, being about 4400 cal (activity of water taken as unity). This value is assumed for the solvolysis of the thiol esters used here. It must be emphasized that errors in this free-energy term, although affecting the absolute magnitude of the rates with amides, do not affect their relative rates and, hence, the β values in the linear free-energy relationships.

(11) B. A. Cunningham and G. L. Schmir, *J. Amer. Chem. Soc.*, **88**, 551 (1966); **89**, 917 (1967).
(12) R. K. Chaturvedi, A. E. McMahon, and G. L. Schmir, *ibid.*, **89**, 6934 (1967).
(13) G. L. Schmir, *ibid.*, **90**, 3478 (1968).
(14) R. E. Barnett and W. P. Jencks, *ibid.*, **91**, 2358 (1969).
(15) L. V. Kozlov and L. M. Ginodman, *Biokhimiya*, **30**, 1051 (1965).
(16) J. J. O'Neill, H. Kohl, and J. Epstein, *Biochem. Pharm.*, **8**, 399 (1961); W. P. Jencks, S. Cordes, and J. Carriuolo, *J. Biol. Chem.*, **235**, 3608 (1960); S. S. Tate and S. P. Datta, *Biochem. J.*, **94**, 470 (1965); W. S. Sly and E. R. Stadtman, *J. Biol. Chem.*, **238**, 2639 (1963); R. H. Himes and J. C. Rabinowitz, *ibid.*, 237, 2903 (1962).

Schmir[13] has given an equation for the pH–rate profile of an amide alcoholysis, but this is not based on absolute values.

Results and Discussion

The neutral alcoholysis of thiolysis of amides may be formally written as

$$RXH + >N-\overset{\overset{O}{\|}}{C}-R' \underset{k_r}{\overset{k_f}{\rightleftharpoons}} RX-\overset{\overset{O}{\|}}{C}-R' + >NH \qquad (4)$$

Mechanistically, however, there may exist kinetically equivalent schemes involving, or not involving as the case may be, intermediates, between which schemes there is no *a priori* distinction. A simple case is given in eq 5. If the equilibrium constant for eq 4 is K_e, then

$$RX^- + H^+ + >NC(=O)R' \underset{k_r}{\overset{k_f^1}{\rightleftharpoons}} RXC(=O)R' + >NH \qquad (5)$$

the microscopic rate constants are related to each other by eq 6 and 7, where K_a is the ionization constant for

$$k_f = K_e k_r \qquad (6)$$

$$k_f^1 = (K_e k_r / K_a) \qquad (7)$$

RXH. A similar formal process occurs for the reaction of the proteolytic enzymes chymotrypsin and papain with amides.

$$\text{chymotrypsin–OH} + >NC(=O)R \rightleftharpoons \text{chymotrypsin–O–C(=O)–R} + >NH \qquad (8)$$

$$\text{papain–SH} + >NC(=O)R \rightleftharpoons \text{papain–S–C(=O)–R} + >NH \qquad (9)$$

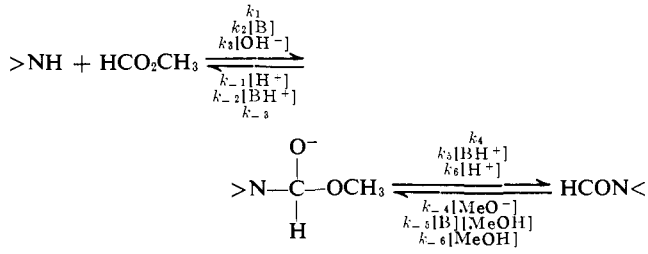
Similar formal schemes may be written for the alcoholysis and thiolysis of esters.

Acetic acid has been chosen as the substrate for the reactions of esters as the experimental data are available concerning this. It was shown in ref 1 that acetic acid reacts as a typical ester—hydroxyl acetate. However, the calculations in that paper concerning the rate constants for nucleophilic attack on acetic acid are all too low by a factor of 55.5 due to the incorrect use of a standard state of 55.5 *M* for water. It must be emphasized that the β value for the attack of nucleophiles on acetic acid remains unaltered as this is a measure of relative rate constants. Comparison of these rate constants with those for alkyl acetates[1] shows that in reactivity acetic acid is equivalent to an acetate ester of an alcohol of pK_a 16.4. The thermodynamic stability is 0.9 kcal less than predicted from the linear free-energy relationship of Gerstein and Jencks[8] and acetic acid has a "free energy of hydrolysis" expected for an acetate of an alcohol of pK_a 15.

In an important study, Blackburn and Jencks elegantly demonstrated that the aminolysis of methyl

formate is consistent with Scheme I.[3] Furthermore, they accurately determined the values of k_1, k_2, and the

Scheme I. Methanolysis of Formamides



ratios k_{-1}/k_{-4} and k_{-2}/k_4 for a series of amines of varying pK_a, k_3 for the morpholine reaction, and the ratios k_5/k_4 and k_6/k_4 for the hydrazinolysis.

The transition states for the aminolysis reactions are the same as those for the alcoholysis reactions. This application of the principle of microscopic reversibility and the calculation of reverse direction rate constants from equilibrium constants and forward direction rate constants enable us to discuss fully the alcoholysis reactions. The calculated rate constants for the reactions of morpholine and hydrazine are given in Table I, along with the experimental rate constants of Blackburn and Jencks.

Table I. Methanolysis of Formamides at 25° [a,b]

Rate constant	Formylmorpholine[c]	Formylhydrazine[d]
k_1, M^{-1} sec^{-1}	4.5×10^{-3}	8.3×10^{-2}
k_2, M^{-2} sec^{-1}	6.5×10^{-2}	2.8
k_3, M^{-2} sec^{-1}	25	
k_{-1}/k_4, M^{-1}	2.3×10^{7}	5.5×10^{8}
k_{-2}/k_4, M^{-1}	0.47	90
k_5/k_4, M^{-1}		1.2
k_{-3}/k_4	1.28×10^{-3}	
k_{-4}, M^{-1} sec^{-1}	0.34[e]	0.57[e]
k_{-5}, M^{-2} sec^{-1}		4.3×10^{-8} [e]
k_{-6}, M^{-1} sec^{-1}		4.3×10^{-10} [e]

[a] Except where noted rate constants are from G. M. Blackburn and W. P. Jencks, *J. Amer. Chem. Soc.*, **90**, 2638 (1968). [b] Ionic strength maintained at 1.5[a]. [c] pK_a of morpholine is 8.84.[a] [d] pK_a of hydrazine is 8.30.[a] [e] Calculated in this study.

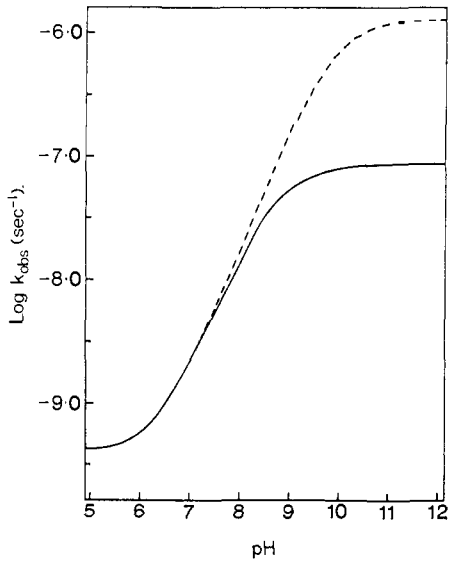
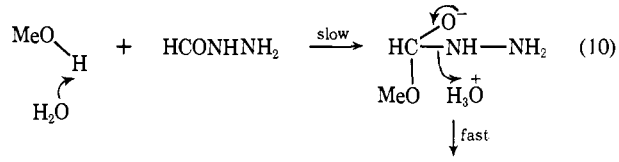


Figure 1. The pH-rate profile for the methanolysis of formylhydrazine at 25°, calculated from the equilibrium constants of this study and ref 6 and the rate constants of ref 3. The broken line represents the calculated influence of the addition of 1 M total concentration of a hypothetical general base of pK_a 7. The upper plateaus are the mechanistically nucleophilic attack of methoxide ion followed by general acid catalyzed rate-determining breakdown of a tetrahedral intermediate.

pH-Rate Profile for the Methanolysis of Formylhydrazine. Two reactions formally representing eq 4, that is kinetically the reaction of MeOH with formylhydrazine, exist in the pH region 5–12, as shown in Figure 1. These are the plateaus in Figure 1 below pH 5.5 and above 10. Superimposed on the high pH plateau should be a reaction involving methoxide ion attack, but the data are not available concerning this.

The low-pH plateau is the reaction of undissociated methanol, involving rate-determining formation of the tetrahedral intermediate.



(10)

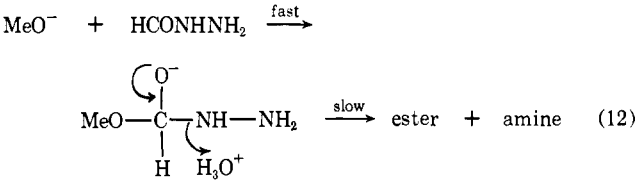
The tetrahedral intermediate breaks down preferentially to give ester and amine. The rate constant for this process is 4.3×10^{-10} M^{-1} sec^{-1}. This reaction is subject to general base catalysis; a two-point Brønsted plot based on water and hydrazine as the general bases gives a β value of 0.36. Significantly the attack of methoxide ion on the amide has a high positive deviation from this plot indicating that this reaction is true nucleophilic attack and not the kinetically equivalent mechanism of hydroxide ion catalyzed methanol attack, [HO$^-$][MeOH]. Analogous behavior occurs for the general base catalyzed attack of water on the ester carbonyl group; β values of 0.3–0.5 are found with high positive deviations for the hydroxyl group.[17,18]

Between pH 6 and 9 the reaction changes from rate-determining attack of methanol to methoxide ion, the rates of the methoxide ion and methanol reactions being equal at pH 6.4, 9.1 units below the pK_a. A similar result was found for the methanolysis of acetylpyridinium ions,[1] where the nucleophilic rates of methoxide and methanol are equal at roughly pH 7.0. The nucleophilic reactivity of methoxide ion is some 10^9-fold greater than that of water-catalyzed methanol attack.

The rate constants k_{-4} for methoxide ion attack on various formamides of alkylamines and hydrazine are empirically found to fit eq 11. The reaction rate is enhanced by electron withdrawal in the amine portion, stablizing the anionic transition state.

$$\log k_{-4} = 7.2 - 0.67pK_{a_{>NH}},\ M^{-1}\ \text{sec}^{-1} \qquad (11)$$

At high pH, the plateau represents rate-determining breakdown of the tetrahedral intermediate thus



(12)

(17) W. P. Jencks and J. Carriuolo, *J. Amer. Chem. Soc.*, **83**, 1743 (1961).

(18) A. R. Fersht and A. J. Kirby, *ibid.*, **89**, 4857 (1967).

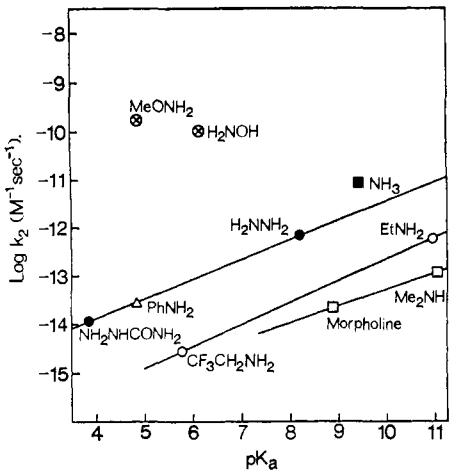


Figure 2. Plots of the logarithms of the second-order rate constants for the reaction of phenol with various acetamides against the pK_a of the leaving group amine, at 25°. Data are from Table II.

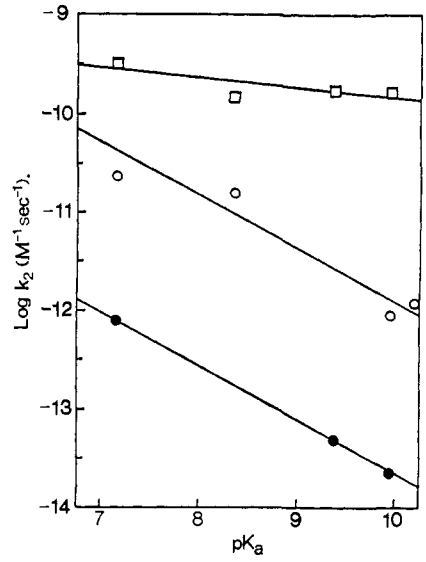


Figure 3. Plots of the logarithms of the second-order rate constants for the reactions of various phenols with acetylmorpholine (●), acetylhydrazine (○), and acetylmethoxyamine (⊡), against the pK_a of the phenol, at 25°. Data are from Table III and for the rate law depending on [phenol][amide].

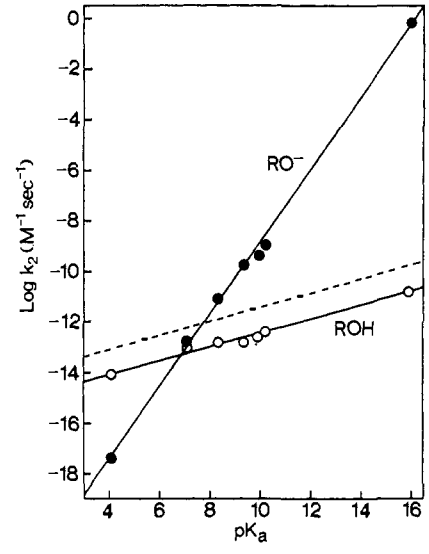


Figure 4. Plots of the logarithms of the second-order rate constants for the reactions of alkoxide (phenoxide) and alcohols (phenols) with acetic acid to form esters, against the pK_a of the alcohol (phenol) at 25°. Data are from Table V. The dashed line represents the effect of a 1 *M* hypothetical general base of pK_a 7 on the alcohol (phenol) reaction.

The reaction is mechanistically nucleophilic attack followed by general acid catalysis by the proton. Other general acids, including water, may take the place of the proton in the slow step. Kinetically, this is equivalent to general base catalysis by the conjugate base. The α value for general acid catalysis is 0.7, or written as general base catalysis of methanol attack the β value is 0.3.[3]

The effect of a hypothetical general base of pK_a 7.0 is shown in Figure 1. The catalysis is manifested at high pH, where mechanistically, the *acid* form of the base is the reactive catalyst for the slow step in eq 12.

(The low-pH region of methanol rate-determining attack is not effectively catalyzed, the general base being mainly protonated, and the small amount of reactive species being swamped by the water catalysis.)

The observed rate constant, based on methanol concentration, for the high pH plateau is 9×10^{-8} M^{-1} sec^{-1}, expressed as eq 4. A 1 *M* solution of general base of pK_a 7.0 increases this rate constant to 1.3×10^{-6} M^{-1} sec^{-1}.

The general approximate empirical equation for the high-pH plateau region for amine variation in the methanolysis of formamides is, for the non-α-effect amines

$$\log k_{obsd} = -11 + 0.2pK_{a_{>NH}},\ M^{-1}\ sec^{-1} \quad (13)$$

The positive β value reflects the demand for acid catalysis. Sufficient data are not available to calculate the β value for the low-pH region. However, rate-determining attack by methanol should involve a negative β value, similar to the −0.67 for the methoxide ion attack k_{-1} (see eq 11).

Formylhydrazine is more reactive than the amides of the non-α-effect amines due to favorable partition of the tetrahedral intermediate,[3] the rate constant for attack of methoxide on the carbonyl group being about the same as for an equivalent non-α-effect compound. Another corollary of the paper by Blackburn and Jencks is that the changeover from rate-determining formation to breakdown of the tetrahedral intermediates occurs at about 1–2 pH units below the pK_a of the leaving group amine. Below this pH, the attack of alcoholate is rate determining, above this pH, acid-catalyzed expulsion of amine.

The General Alcoholysis and Thiolysis of Amides and Esters. The derived data for the reaction of alcohols and a thiol with amides and an ester are given in Tables II–V and illustrated in Figures 2–4. For the rate law involving [ROH][amide], it is seen that the reactivities of the amides fall into several classes. The acylated hydroxylamines are about $1–5 \times 10^3$ times more reactive than the acyl hydrazines and anilines, which are some 20-fold more reactive than the acyl primary amines which in turn are about four times more reactive than the acyl secondary amines. Acetamide is somewhat more reactive than an acyl hydrazine of

Table II. Alcoholysis and Thiolysis of Acetic Acid Amides at 25–30°

Amide of acetic acid and amine	pK_a[d]	Nucleophile: Phenol[a,b] k_2, M^{-1} sec^{-1}	Nucleophile: 4-Nitrophenol[a,b] k_2, M^{-1} sec^{-1}	Nucleophile: 2,2,2-Trifluoroethanethiol[c] k_2, M^{-1} sec^{-1}
Ethylamine	10.97	5.7×10^{-13}	2.7×10^{-12}	
2,2,2-Trifluoro-ethylamine	5.84	2.7×10^{-15}	5.7×10^{-14}	
Aniline	4.85	2.7×10^{-14}	4.7×10^{-13}	
Hydrazine	8.20	6.6×10^{-13}	2.0×10^{-11}	4.5×10^{-8}
Semicarbazide	3.86	1.2×10^{-14}	1.1×10^{-13}	
Hydroxylamine	6.17	1.1×10^{-10}	5.7×10^{-10}	8.2×10^{-7}
Methoxyamine	4.80	1.7×10^{-10}	3.2×10^{-10}	
Dimethylamine	11.06	1.2×10^{-13}		
Morpholine	8.87	2.2×10^{-14}	8.0×10^{-13}	6.0×10^{-10}
Ammonia	9.45	8.7×10^{-12}	3.2×10^{-11}	

[a] Calculated from rate constants at 25° and ionic strength 1.0, using free energy of hydrolysis of phenyl acetates from J. Gerstein and W. P. Jencks (*J. Amer. Chem. Soc.*, **86**, 4655 (1964)) and calculated free energies of hydrolysis of acetamides (A. R. Fersht and Y. Requena, *ibid.*, **93**, 3499 (1971)). [b] Aminolysis rate constants from W. P. Jencks and M. Gilchrist, *ibid.*, **90**, 2622 (1968), W. P. Jencks and J. Carriuolo, *ibid.*, **82**, 675, 1778 (1960), and L. do Amaral, K.Koehler, D. Bartenbach, T. Pletcher, and E. H. Cordes, *ibid.*, **89**, 3537 (1967). [c] Calculated from rate constants at 30° and ionic strength 1.0 using the aminolysis data of M. J. Gregory and T. C. Bruice (*ibid.*, **89**, 2121 1967)) and assuming a free energy of hydrolysis of 4400 cal for the acetylthiol. [d] At 25° and ionic strength 1.0 from J. Sayer and W. P. Jencks, *ibid.*, **91**, 6353 (1969), W. P. Jencks and M. Gilchrist, *ibid.*, **90**, 2622 (1968), and A. R. Fersht and Y. Requena, *ibid.*, **93**, 3499 (1971).

Table III. Alcoholysis of Amides at 25–30°

Phenol	pK_a	Acetylmorpholine[a] k_2, M^{-1} sec^{-1}	Acetylmethoxyamine[a] k_2, M^{-1} sec^{-1}	Acetylhydrazine[b] k_2, M^{-1} sec^{-1}
4-Methyl-	10.19			1.2×10^{-12}
H-	9.95	2.2×10^{-14}	1.7×10^{-10}	9.1×10^{-13}
4-Chloro-	9.38	5×10^{-14}	1.8×10^{-10}	
3-Nitro-	8.35		1.5×10^{-10}	1.6×10^{-11}
4-Nitro-	7.14	8×10^{-13}	3.2×10^{-10}	2.4×10^{-11}

[a] Calculated from the data of L. do Amaral, K. Koehler, D. Bartenbach, T. Pletcher, and E. H. Cordes (*J. Amer. Chem. Soc.*, **89**, 3537 (1967)), at 25° and ionic strength 1.0. [b] Calculated from the data of T. C. Bruice and S. J. Benkovic (*ibid.*, **96**, 418 (1964)), at 30° and ionic strength 1.0.

Table IV. Reaction of Phenol and the Phenolate Ion with Amides at 25° and Ionic Strength 1.0[a]

Amide	pK_a of amine	PhOH attack k_2, M^{-1} sec^{-1}	PhO$^-$ attack[b] k_2, M^{-1} sec^{-1}
Acetylmorpholine	8.87	2.2×10^{-14}	7.6×10^{-14} [b]
Acetyldimethylamine	11.06	1.2×10^{-13}	6.6×10^{-15} [b]

[a] Calculated from the data of W. P. Jencks and J. Carriuolo, *J. Amer. Chem. Soc.*, **82**, 675 (1960), J. Gerstein and W. P. Jencks, *ibid.*, **86**, 4655 (1964), and A. R. Fersht and Y. Requena, *ibid.*, **93**, 3499 (1971). [b] Rate constant calculated for the presence of 55.5 M water. The third-order rate constant is that factor lower.

Table V. Reaction of Alcohols and Alkoxide Ions with Acetic Acid at 25°

Alcohol	pK_a[a]	ROH reaction k_2,[b] M^{-1} sec^{-1}	RO$^-$ reaction k_2,[c] M^{-1} sec^{-1}
Ethanol	15.9	1.5×10^{-11}	6.6×10^{-1}
4-Methylphenol	10.19	3.5×10^{-13}	1.1×10^{-9}
Phenol	9.95	2.5×10^{-13}	4.2×10^{-10}
4-Chlorophenol	9.38	1.7×10^{-13}	1.5×10^{-10}
3-Nitrophenol	8.35	1.7×10^{-13}	7.8×10^{-11}
4-Nitrophenol	7.14	1.0×10^{-13}	1.7×10^{-13}
2,4-Dinitrophenol	4.11	7.6×10^{-15}	4×10^{-18}

[a] Thermodynamic value for the conjugate acid; from H. A. Sober, Ed., "Handbook of Molecular Biology," Chemical Rubber Publishing Co., Cleveland, Ohio, 1968. [b] Calculated from the data of V. Gold, D. G. Oakenfull, and T. Riley, *J. Chem. Soc. B*, 515 (1968); J. Gerstein and W. P. Jencks, *J. Amer. Chem. Soc.*, **86**, 4655 (1964); J. F. Kirsch and W. P. Jencks, *ibid.*, **86**, 837 (1964). [c] Taken from A. R. Fersht and W. P. Jencks, *ibid.*, **92**, 5442, (1970) (and corrected by a factor of 55). [d] Free energy of hydrolysis estimated by interpolation.[b]

equivalent pK_a. Apart from the hydroxylamines, within a particular class the amides with more basic leaving groups are more reactive. It is also seen (Figure 3) that the more acidic alcohols (phenols) are the more reactive.

The β value for the variation of amine is 0.3–0.5. The variation of phenol nucleophile leads to a β value of −0.4. The acylhydroxylamines are exceptions, but there is the possibility that in the reactions of methoxyamine with phenyl acetates rate-determining proton transfers are important.[19]

A general empirical equation may be set up for alcoholysis using eq 6. The combination of the expressions for the equilibria of ester formation from amides and alcohols[6] with the approximate general equation for aminolysis of phenyl acetates calculated from the data of Jencks and Gilchrist[5] leads to eq 14 for alcoholysis rate constants k. The β values are the com-

$$\log k = -10 + (0.2 - 0.5)\mathrm{p}K_{a_{>NH}} - (0.2 - 0.4)\mathrm{p}K_{a_{ROH}} \quad (14)$$

posites of those for the equilibrium and aminolysis reaction. (However, it has been found[1,2] that the reaction of basic amines with acetate esters of very good leaving groups has a very low sensitivity to both the basicity of the leaving group nucleophile; this leads to β values of approximately +0.70 for alcohol variation and −0.5 for amine variation in the alcoholysis reactions, the β values being of opposite sign to the above case.)

(19) L. do Amaral, K. Koehler, D. Bartenbach, T. Pletcher, and E. H. Cordes, *J. Amer. Chem. Soc.*, **89**, 3537 (1967).

These rate constants are of course derived for the experimental conditions of the aminolysis reactions. These tend to be at pH's around and above the pK_a of the amine involved.

2,2,2-Trifluoroethanethiol (pK_a 7.3[20]) is some thousand times more reactive than *p*-nitrophenol (pK_a 7.1). A β value of +0.4, similar to that for alcoholysis, is calculated (from ref 20) for the thiolysis of amides of varying amine constituents, the more basic amides again being the more reactive.

Little data are available for the reactions of amines with thiol esters of varying thiol constituent. However, the rate constants for the hydrazinolysis of butyl thiolacetate[21] and 2,2,2-trifluoroethyl thiolacetate[20] are available and a tentative β value of −0.9 may be calculated from these results for a "series" of thiols reacting with acetylhydrazine.

Toward acetic acid, *i.e.*, an ester of a poor leaving group, the phenols and alcohols exhibit a β value of 0.3, the more basic hydroxyl compounds being the more reactive; see Figure 4. The reactivity of thiols toward trifluoroacetic acid is in the reverse direction, a β value of −0.4 being calculated.[22]

These results are summarized in Table VI. The trend in reactivity of alcohols toward esters is in the opposite direction as that toward amides. As the nucleophilic alcohol becomes more basic, its reactivity toward esters increases but toward amides decreases.

Table VI. Summary of Structure–Reactivity Relationships for Alcoholysis and Thiolysis[a]

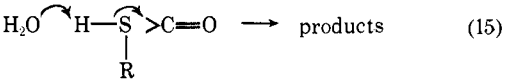
Nucleophile	Substrate	β for variation of— Nucleophile	Leaving group
Alcohols	Amides[b]	−(0.2–0.4)	+(0.2–0.5)
Alcohols	Amides[c]		−0.7
Alcohols	Amides[d]	+0.7	−0.5
Alcohols	Esters	+0.3	−0.4
Chymotrypsin	Anilides[e]		+(0.5–0.7)
Chymotrypsin	Amides[f]		−(0.3–0.4)
Chymotrypsin	Esters[g]		−0.6
Chymotrypsin	Esters[h]		−0.2
Thiols	Amides		+0.4
Thiols	Esters	−0.4	
Papain	Anilides[i]		+(0.3–0.4)
Papain	Esters[h]		−0.6

[a] See text for derivation, symbols, and sources. "Alcohols" is used as a generic term for alcohols, phenols, and water. [b] Mechanism is the rate-determining breakdown of a tetrahedral intermediate for reactions of alcohols; for phenols, may also represent nucleophilic attack on an N-protonated amide. [c] Rate-determining formation of a tetrahedral intermediate. [d] Weak nucleophiles ($pK_a < 5$) and amides of basic amines ($pK_a > 10$). [e] Observed for k_1 and in one case for k_1/K_S. [f] Calculated for k_1/K_S from k_{-1} and equilibrium constants. [g] Observed for k_1/K_S with alkyl esters. [h] Observed for k_1/K_S and aryl esters. [i] Observed for k_1.

The relative reactivities of the ionized and un-ionized alcohols toward the amides and acetic acid are of great interest. In some cases, the un-ionized alcohol appears to be more nucleophilic than the fully formed alkoxide ion (Tables IV and V and Figure 4).

Mechanisms. We are concerned with the broader aspects of the mechanisms of reactions of thiols and alcohols with esters and amides as the nature of the transition states in the alcoholysis or thiolysis reactions has been studied in the reverse reactions, the aminolysis and hydrolysis of esters. The main question to be answered concerns the state of the reagents prior to reaction, for example, whether the thiolysis reaction is initiated by the thiolate anion or the undissociated thiol. In many cases the situation is still not totally clear. For example, it has been suggested that the aminolysis of phenyl acetates occurs *via* rate-determining formation of tetrahedral intermediates.[2,20] However, we have recently shown that the supporting arguments for this are ambiguous.[1] To simplify discussion, if tetrahedral intermediates exist and their formation is not rate determining, the rate-determining step will be referred to in the genetic term of rate-determining breakdown even if the rate-determining step is the protonic interconversion of one intermediate to another.

Thiolysis of Esters. Ethanethiol and ethyl mercaptoacetate react with trifluoroacetic acid with a β value of −0.4, calculated from the data of Barnett and Jencks.[22] Two classes of mechanism are possible: eq 15, the general base catalyzed attack of the undissociated thiol, or eq 16, the reaction of the thiolate anion combined with a protonation step. On general grounds



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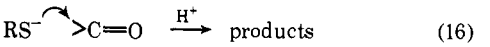
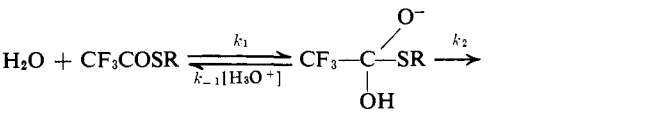


(16)

there is a notable lack of example of effective general base catalysis of thiol attack.[14,23–28] Convincing studies from the laboratories of Bender,[29] Bruice,[30] and Jencks[17] demonstrate that the hydrolysis of the trifluoroacetic acid esters of the two mentioned thiols is consistent with the following scheme (eq 17).



(17)

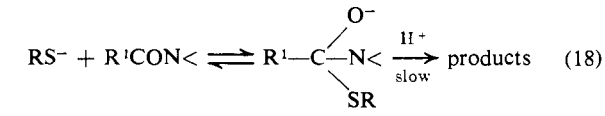
The mechanism is unsymmetrical, the expulsion of the hydroxyl, but not that of the thiol, from the tetrahedral intermediate being acid catalyzed. Application of the principle of microscopic reversibility requires that under the same conditions the thiolysis of the acid must occur by attack of the *thiolate anion*. The reaction of ethanethiol with trifluoroacetic acid[21] involves rate-determining attack of the anion below pH 2. Between pH 2 and 6 the reaction proceeds by preequilibrium addition of the thiolate anion to the carbonyl group, followed by rate-determining specific acid catalyzed expulsion of water. Above pH 6, the rate-determining step is the expulsion of hydroxide ion from the intermediate.

The important conclusion from this is that even at 10 pH units below its pK_a, ethanethiol is less nucleophilic than the thiolate anion, despite the latter's presence in only one part in 10^{10}.

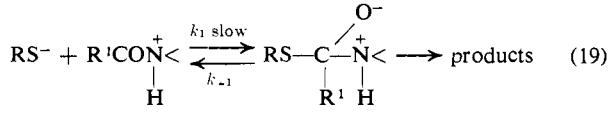
(20) M. J. Gregory and T. C. Bruice, *J. Amer. Chem. Soc.*, **89**, 2121 (1967).
(21) L. R. Fedor and T. C. Bruice, *ibid.*, **86**, 4117 (1964).
(22) R. E. Barnett and W. P. Jencks, *J. Org. Chem.*, **34**, 2777 (1969).
(23) W. P. Jencks and J. Carriuolo, *J. Biol. Chem.*, **234**, 1280 (1959).
(24) J. R. Whitaker, *J. Amer. Chem. Soc.*, **84**, 1900 (1962).
(25) J. W. Ogilvie, J. T. Tildon, and B. S. Strauch, *Biochemistry*, **3**, 754 (1964).
(26) J. F. Kirsch and M. Igelström, *ibid.*, **5**, 783 (1966).
(27) G. E. Lienhard and W. P. Jencks, *J. Amer. Chem. Soc.*, **88**, 3982 (1966).
(28) R. E. Barnett and W. P. Jencks, *ibid.*, **89**, 5963 (1967).
(29) M. L. Bender and H. d'A. Heck, *ibid.*, **89**, 1211 (1967).
(30) L. R. Fedor and T. C. Bruice, *ibid.*, **86**, 5697 (1964).

Thiolysis of Amides. The reactivity of thiols toward amides is increased by increasing basicity of the amide and increasing acidity of the thiol as indicated by the β values in Table VI. The mechanism of thioester aminolysis is complex. Tetrahedral intermediates have been kinetically detected,[14,31] and examples of their rate-determining formation, breakdown, and protonic interconversion demonstrated.[14,31]

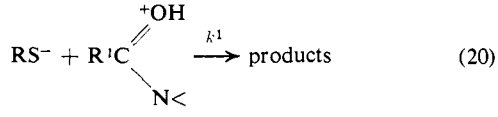
The important conclusion from the last section concerning the nucleophilicities of the thiol and thiolate anions eliminates consideration of the former, leaving two reasonable classes of mechanism (eq 18-20). The



(18)



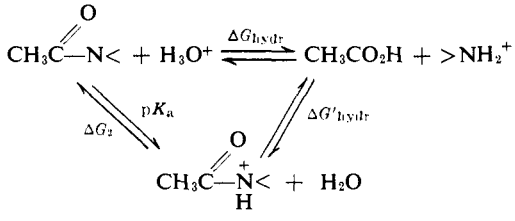
(19)



(20)

second pair of mechanisms involves attack on the pre-protonated amide; the first mechanism involves attack on the amide followed by proton transfer. Controversy has raged over the position of protonation of amides, but there is now overwhelming evidence in favor of O protonation, as in eq 20, being the rule.[32] There is little information concerning the pK_a's for N protonation of representative amides as the experimentally determined values are the basicities for the O protonation, and thus, those for the N protonation are experimentally inaccessible. To assess the feasibility of the mechanism involving pre-rate-determining step N protonation, the relevant pK_a is required and we have devised the following scheme for its estimation.

Estimation of pK_a for N Protonation of Amides. The pK_a for N protonation of amides may be estimated from the following thermodynamic cycle



The free energies of hydrolysis of amides are now known,[6] but not those of protonated amides. However, as the protonated amide is equivalent to an acylated tertiary amine, we make the assumption that the $\Delta G'_{hydr}$ is the same as for an acetylpyridinium ion of a pyridine of equivalent pK_a. For acetylpyridinium ions[33] (in kilocalories)

$$\Delta G_{hydr} = -22.31 + 0.75pK_{a_{>NH}} \quad (21)$$

and for amides[1]

$$\Delta G_{hydr} = 0.41 - 0.66pK_{a_{>NH}} \quad (22)$$

so that

$$pK_a = -16.6 + 1.04pK_{a_{>NH}} \quad (23)$$

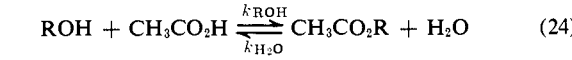
These estimates give minimum values for the ionization constants as resonance stabilization in the pyridinium compounds and solvation energy differences are ignored. The estimated pK_a for N protonation of *N*-acetylmorpholine is -7.4. The experimental pK_a's for O protonation are of the order of 0 (see Appendix).[32]

The specific rate constant, k, required to account for the observed rate constant (6.0×10^{-10} sec^{-1}) of the reaction of 2,2,2-trifluoroethanethiol with *N*-acetylmorpholine according to mechanism 19 is 3×10^5 M^{-1} sec^{-1}. This is not impossibly high considering the high reactivity of acyl tertiary amines.[1] The rate constant for the transfer of a proton from water to an acid of pK_a -7.4 is about 10^{-11} sec^{-1},[34] and that from solvated proton about 10^5 M^{-1} sec^{-1}. Clearly the rate of formation of an N-protonated amide *via* proton transfer from water is not consistent with mechanism 19. Pre-equilibrium protonation from the proton is possible though.

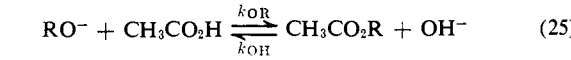
The required specific rate constant, k^1, to account for the observed rate in terms of mechanism 20, is about 0.24 M^{-1} sec^{-1}, and again this is feasible. The intramolecular acyl transfer in *S*-acetylmercaptoethylamine[14] does not involve mechanism 19 as the step equivalent to k_{-1} is not rate determining in that aminolysis reaction. Both mechanisms 18 and 20 probably occur, the former being likely at higher pH and the latter at lower pH.

Nucleophilicities of RO^- and ROH. It is commonly argued that the rate constant for the (general base catalyzed) reaction of a protonic nucleophile, such as an alcohol, cannot exceed that for the reaction of the fully formed ion. However, it is seen from Tables IV and V and Figure 4 that this is not necessarily so, or more precisely, that the rate dependent on [ROH] may be greater than that dependent on [RO^-]. These cases of apparently anomalous reactivity reflect the requirement of concurrent acid catalysis for the expulsion of poor leaving groups. Quantitative expressions for the relative reactivities may be developed.

(a) Esters. For acetate ester hydrolysis



(24)



(25)

it follows that

$$k_{ROH}/k_{H_2O} = (k_{OR}/k_{OH})(10^{15.74} - pK_{a_{ROH}}) \quad (26)$$

where 15.74 is the pK_a for the ionization of water. Analysis of available experimental data[2,35,36] gives the following empirical equations for the rate constants (M^{-1} sec^{-1}) of the hydroxide and water catalysis of the

(31) G. M. Blackburn, *Chem. Commun.*, 249 (1970).
(32) E. M. Arnett, *Progr. Phys. Org. Chem.*, 1, 270 (1963); R. J. Gillespie and T. Birchall, *Can. J. Chem.*, 41, 148 (1963); T. Birchall and R. J. Gillespie, *ibid.*, 41, 2642 (1963).
(33) A. R. Fersht and W. P. Jencks, *J. Amer. Chem. Soc.*, **92**, 5432 (1970).
(34) M. Eigen and L. de Maeyer, *Tech. Org. Chem.*, **8** (2), 1031 (1963).
(35) V. Gold, D. G. Oakenfull, and T. Riley, *J. Chem. Soc. B*, 515 (1968).
(36) T. C. Bruice, T. H. Fife, J. J. Bruno, and N. E. Brandon, *Biochemistry*, **1**, 7 (1962).

hydrolysis of acetate esters

$$\log k_{H_2O} = -5.08 - 0.39pK_{a_{ROH}} \quad (27)$$

$$\log k_{OH} = 2.67 - 0.23pK_{a_{ROH}} \quad (28)$$

The alcoholysis rate constants are thus calculated to obey eq 29

$$\log k_{OR} = \log k_{ROH} + 1.16pK_{a_{ROH}} - 7.99 \quad (29)$$

k_{OR} is equal to k_{ROH} for an alcohol of pK_a 6.90. The pH at which the *rates* of nucleophilic displacement by the alkoxide ion and alcohol are equal, *i.e.*, $k_{ROH}\cdot[ROH] = k_{OR}[RO^-]$, is given by

$$pH = 7.99 - 0.16\ pK_{a_{ROH}} \quad (30)$$

This is also the pH at which the hydroxide and water hydrolysis rates $k_{OH}[OH^-]$ and $k_{H_2O}[H_2O]$ of the ester are equal.

The presence of a general base of pK_a 7.0 will favor the attack of the alcohol and for pH's greater than 7 will increase the crossover pH by about 1 unit.

(b) Amides. The data from a series of papers by Jencks[2,33,37] on the aminolysis of phenyl acetate and methyl formate by amines in the pK_a range 8.3–11.4 fit well the empirical eq 33. As the equilibrium constants

$$RO^- + H_2O + CH_3CON< \underset{k_{OH}}{\overset{k_{OR}}{\rightleftharpoons}} CH_3CO_2R + OH^- + >NH \quad (31)$$

$$ROH + CH_3CON< \underset{k_{H_2O}}{\overset{k_{ROH}}{\rightleftharpoons}} CH_3CO_2R + >NH \quad (32)$$

$$\log k_{OH} = \log k_{H_2O} + 15.34 - 1.0pK_{a_{>NH}} \quad (33)$$

for reactions 31 and 32 are related by just the difference in the pK_a of water and alcohol as for eq 24–26, it is easily shown that

$$\log k_{OR} = \log k_{ROH} + pK_{a_{ROH}} - pK_{a_{>NH}} - 0.4 \quad (34)$$

The relative nucleophilities of the alcohol and alkoxide ion are governed by the difference in pK_a (+0.4) of the nucleophile and leaving group amine. When the pK_a of the alcohol is greater than that of the amine (+0.4), the alkoxide is more nucleophilic, and for the opposite pK_a difference the alcohol is the more effective nucleophile. The relative rates of the reactions of $k_{OR}[RO^-]$ and $k_{ROH}\cdot[ROH]$ obey eq 35. That is, at pH's above the pK_a

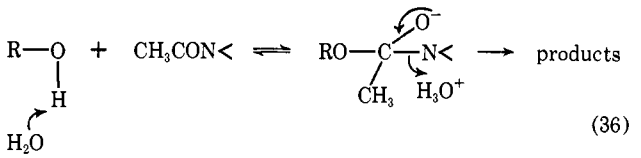
$$\log k_{OR}[RO^-] - \log k_{ROH}[ROH] = pH - pK_{a_{>NH}} - 0.4 \quad (35)$$

(+0.4) of the amine leaving group, the reaction pathway occurs mainly by attack of the alkoxide ion and at low pH's by the alcohol reaction.

It must be emphasized that the data available are for alcohols of pK_a 10–16 and amines 8.3–11.4 and the relationships may not hold outside this range.

A general base of pK_a 7 at 1 *M* concentration should increase the efficiency of the alcohol reaction tenfold, raising the crossover pH's by 1 unit.

Nature of the ROH Reaction. The problem arises as with the thiolysis reaction as to which of the kinetically equivalent reactions constitutes the major reaction pathway. Again, one cannot distinguish between the



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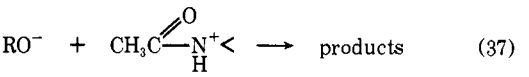

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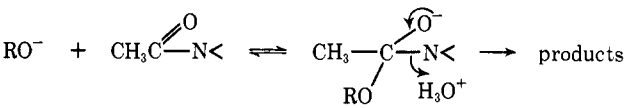


(38)

mechanisms by the qualitative use of parameters which are quasiequilibrium in nature, depending on differences between transition and ground states such as β values, salt, solvent, and isotope effects, and enthalpies and entropies of activation.[38] These quantities are essentially equilibrium phenomena and are independent of the route of the reaction. It is only when a kinetic feature is involved that differentiation is possible. Examples of this are observed changes in the rate-determining step and calculations that show that an intermediate step requires a rate constant greater than diffusion control or that a negative enthalpy of reaction is required for a kinetic step to account for the overall observed rate or enthalpy change.

It is seen from Table IV that phenol appears to be more reactive toward acetylmorpholine than is phenolate ion; at a pH equal to the pK_a of phenol, 95% of the reaction is still *via* the phenol reaction. The mechanism cannot be that of eq 36 involving simple rapid reversible general base catalyzed addition of phenol followed by rate-determining breakdown, as the phenolate ion would be more nucleophilic than general base catalyzed attack of phenol, and eq 38 would be preferred. If, however, (36) is a "one-encounter"[39] mechanism, the catalyzing water molecule in the first step being also the catalyzing acid in the second step, not diffusing out of the encounter complex but giving an artifically high local concentration of protons, then perhaps this could account for a faster rate in eq 36 than in eq 38? This seems unlikely. The tetrahedral intermediate in eq 36 would partition favorably to give products,[3,5] so that the *first* step would be rate determining. This would require a *negative* β value, as observed for rate-determining attack of methoxide on formylhydrazine, but a positive β value is found.

At higher pH mechanism 36 is unlikely. By analogy with the methanolysis of formylhydrazine it could be important at low pH however. The transition between the two mechanisms for this methanolysis is some 9 pH units below the pK_a of methanol.

We prefer eq 38 as being the usually encountered mechanism for alcohols with the possibility that eq 37 (or a mechanism involving O protonation as in eq 20) may be operative in the case of phenols. Mechanism 37 cannot be ruled out by the high rate constants involved. The required specific rate constant calculated

(37) W. P. Jencks and M. Gilchrist, *J. Amer. Chem. Soc.*, **88**, 104 (1966).

(38) An informative discussion is given in W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, pp 603–605.

(39) See ref 38, p 211.

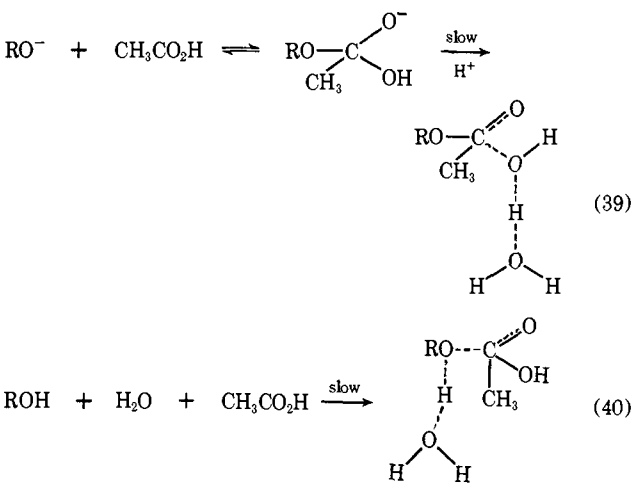
for mechanism 37 involving phenolate ion and N-protonated acetylmorpholine is $5 \times 10^3\ M^{-1}\ \text{sec}^{-1}$, a value well within the known reactivity of acylated tertiary amines.[1] The aminolysis of phenyl acetate shows general base catalysis by a second molecule of amine or hydroxide ion[2] and at first sight this is easier to incorporate in the mechanism 38 which involves *general* acid catalysis rather than eq 37 which involves specific acid. For the reaction of more acidic alcohols eq 37 is possible as the aminolysis of the activated phenyl acetates is not subject to general base catalysis.

Pertinent to this is the relative reactivity toward esters of tertiary to primary or secondary amines. The tertiary amines cannot be activated by water general base catalysis and these reactions are models for unassisted nucleophilic attack in aminolysis and hence also models for the reverse reactions, specific acid catalyzed alcoholysis of amides, mechanism 38. Toward alkyl esters, the tertiary amines are calculated to be some five–six orders of magnitude less reactive than their primary or secondary counterparts.[1] Toward phenyl and 4-nitrophenyl acetate,[5] the tertiary amines quinuclidinol and triethylenediamine are about 20 times less reactive than equivalent primary or secondary amines, but this reactivity difference disappears with 2,4-dinitrophenyl acetate and more activated esters.[1,5] This measure of the relative activation or general base catalysis by water is a possible indication of the relative importance of mechanisms 37 and 38 for alcoholysis. This is strong evidence in favor of mechanism 38 being operative in the reactions of alcohols with amides. It is not necessarily evidence for eq 37 being the mode of reaction of phenols with amides, as mechanism 38 involving the expulsion of a nearly completely protonated amine from the tetrahedral intermediate as the rate-determining step is also consistent with the above.

In summary, the reaction of basic alcohols with amides is satisfactorily described by mechanism 38 and the reaction of phenols is consistent with either a change from mechanism 38 to 37 or a change in nature of mechanism 38.

One further piece of evidence for rate-determining (or partially rate-determining) breakdown here that may find use as a general criterion for position of the rate-determining step is that the hydrazides are more reactive than acetylated primary and secondary amines. The enhanced reactivity in the methanolysis of formylhydrazine was shown to be a consequence of the favorable breakdown of the tetrahedral intermediate rather than the formation step which has a normal rate constant. For mechanisms involving rate-determining formation of tetrahedral intermediates, it is predicted that hydrazides should have no enhanced reactivity.

The description of the alcoholysis of esters is even more difficult to analyze. The problem is again that the β values are sometimes a weak criterion of mechanism. It is a popular fallacy[40] that small β values are indicative of small *bond* changes. They are in fact indicative of small changes of *charge* at the reaction center, which can of course be caused by large but compensating bond changes. For example, consider the two mechanisms 39 and 40, where the dotted lines are indicative of bond making and breaking. Equation 39 involves rate-determining breakdown of an intermediate with a late transition state. Mechanism 40 involves an early transition state with rate-determining formation. The



β value for the loss of a proton from an alcohol is -1, but that for the gain of acyl group is $+1.7$.[8] In eq 39, the loss of the proton is easily compensated by the incipient acyl transfer leading to a low observed β value. In eq 40, compensating simultaneous bond changes cause a low β value. Both mechanisms 39 and 40 are consistent with the observed β of $+0.3$. Two observations are relevant. First, it is seen from Tables II and V that the ester is only as reactive as an amide, despite the usual higher reactivities of esters,[41] for the alcohol reaction. This reflects the lower basicity of oxygen as compared with nitrogen[32] and the corresponding lowering of the efficiency of acid catalysis. Second, it is difficult for a weakly basic nucleophile to displace a highly basic nucleophile from a tetrahedral intermediate. The attack of a strong base such as ethoxide ion has little advantage in having a second step of acid catalysis as in eq 39. The reaction of ethanol with acetic acid is likely to represent classical general base catalysis as in eq 40. On the other hand, the 2,4-dinitrophenol reaction almost certainly represents eq 39, unless a "one-encounter" mechanism is involved, although this was discarded for the amide reactions. However, at some low pH, when the concentration of anions becomes too low, a slower classical general base catalyzed mechanism could come into play. The dividing line between the general base and the anion-specific acid reaction remains to be elucidated. It is further complicated in that in Figure 4 all the points may be fitted to one straight line, a test often applied for constancy of mechanism along a series—but this test has proved to be illusory on occasion.[35] The ethanol reaction is highly symmetrical due to the similar pK_a values of entering and leaving groups and, if the addition of ethanol is general base catalyzed, then the expulsion of hydroxyl from the tetrahedral intermediate will be acid catalyzed as in the slow step of eq 39. Mechanism 40 does not imply that the subsequent hydroxyl expulsion is not catalyzed.

Application to Enzyme Systems. It is generally accepted that the hydrolysis of esters and amides by papain[42] and chymotrypsin[43] follows the minimal reac-

(40) J. E. Reimann and W. P. Jencks, *J. Amer. Chem. Soc.*, 88, 3973 (1966).

(41) M. L. Bender, *Chem. Rev.*, 60, 53 (1960).
(42) G. Lowe, *Phil. Trans. Roy. Soc. London, Ser. B*, 257, 237 (1970).
(43) M. L. Bender and F. J. Kezdy, *J. Amer. Chem. Soc.*, 86, 3704 (1964).

tion Scheme II, where YH is an alcohol of amine. In

Scheme II

$$\mathrm{EXH} + \mathrm{RCOY} \overset{K_s}{\rightleftharpoons} \mathrm{EXH\text{-}RCOY} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{EXCOR} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{EXH} + \mathrm{RCO_2H}$$

$$\pm\mathrm{YH}$$

$$\mathrm{EXH\text{-}RCO_2H} \quad K_p$$

α-chymotrypsin -XH is the hydroxyl of a serine (Ser-195), and in papain -XH is the sulfhydryl of a cysteine (Cys-25). There are some anomalies for the case of chymotrypsin with amides.[44,45]

Use may be made of the Haldane relationship[46] for the overall equilibrium between substrate and products to give a useful equation. If there is a series of amides or esters in which the acyl portion is constant and only the amine or the alcohol, YH, is varied, k_2, k_{-2}, and K_p are constant. The combination of the equations for the hydrolysis equilibria for esters[43] and amides[6] with the Haldane relationship gives for esters

$$\log (k_1/K_s) = \log k_{-1} - 0.70\mathrm{p}K_{a_{ROH}} + \alpha \quad (41)$$

and for amides

$$\log (k_1/K_s) = \log k_{-1} - 0.51\mathrm{p}K_{a_{>NH}} + \beta \quad (42)$$

where α and β are constants defined by the absolute values of the equilibrium constants between substrates and products. Equations 41 and 42 may be used to test the validity of Scheme II by measuring k_{-1} and (k_1/K_s). It is also useful for determining linear free-energy relationships for one of the rate constants from a knowledge of those for the other.

Chymotrypsin. Two important residues at the active site of chymotrypsin are a serine (Ser-195) which may be acylated and a histidine (His-57). In the crystal at pH 4.2 the imidazole of the histidine and the hydroxyl of the serine appear to be H bonded.[47]

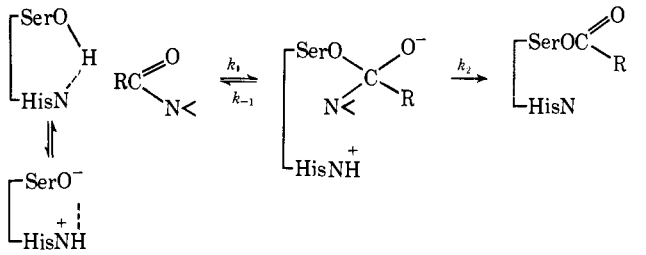
Acylation of Chymotrypsin by Amides. Inward and Jencks[48] have shown that deacylation (k_{-1}) of furoyl chymotrypsin by a series of amines, of pK_a 4.6–11.6, has low sensitivity to the amine pK_a, the β value being in the range + 0.1 to + 0.2. This may be compared with the β of −0.1 to −0.2 we have calculated for the aminolysis of methyl formate involving rate-determining breakdown of the tetrahedral intermediate (with methoxide expulsion), and the value of +0.7 found for the rate-determining attack of amines on methyl formate[3] and the values of +0.8 to +1.0 for the aminolysis of phenyl acetates.[4,5] The β value for k_{-1} requires a β of −0.3 to −0.4 for the variation of (k_1/K_s) for the hydrolysis of the amides of furoic acid. No such studies have been made due to the slow rates that would occur. However, in apparent contradiction, anilides of specific substrates have been examined[49–52] and it is found that k_1 exhibits a positive β value of +0.6 to 0.7, the more basic anilides being more reactive, although the values of k_1/K_s are too scattered to analyze.

This observation that electron withdrawal in anilides decreases k_1, in contrast with the opposite observation for esters, has elicited surprise in the past, but we have now shown this to be a feature also common to the nonenzymatic reactions and not an enzymatic peculiarity.

To account for the positive β value for k_1, Wang and Parker[51,53] have proposed that the acylation step takes place by a mechanism similar to eq 37, a preequilibrium protonation of the amide leaving group nitrogen. In order to obtain a proton transfer rate fast enough to be compatible with the observed catalytic constant it was postulated that a proton was transferred from Ser-195 to the amide *via* directed H bonds with the rapid rates encountered for proton transfer in ice. On the basis of a pK_a for N protonation of anilides of about 0, a just feasible rate was calculated. However, our estimate of the pK_a for N protonation of such an anilide is about −10. As proton transfer rates from weak acids to weak bases are proportional to the ratio of ionization constants, the calculation of Wang and Parker is an overestimate by ten orders of magnitude. Such a mechanism may be discounted, the proton transfer rate to the substrate being too slow by a factor of 10^{10}.

Caplow[52] has suggested that the anilide hydrolysis by chymotrypsin represents rate-determining breakdown of a tetrahedral intermediate. The β values for the reaction are consistent with this. The β values of Inward and Jencks[48] for the reactions of more basic amides are more consistent with the rate-determining formation of a tetrahedral intermediate. Blackburn and Jencks[3] have shown that for the aminolysis of methyl formate the more basic amines are more susceptible to acid-catalyzed expulsion from the tetrahedral intermediate. These observations are consistent with Scheme III.

Scheme III



The β value for the enzymatic hydrolysis of amides is between those found for rate-determining formation, and for the determining breakdown of the tetrahedral intermediate in nonenzymatic systems, but numerically closer to the former, a strict analogy suggests that in the enzyme the two steps are similar in energy and the rate-determining step is not unique. For the more basic amines, k_1 has a tendency to be rate determining; for the less basic amides such as anilides, k_2 tends to be rate determining. The results of the nonenzymatic studies show that either the zwitterion or the H-bonded pair may initiate the reaction, and it is not possible to give an *a priori* distinction between the two.

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(45) R. M. Epand, *Biochem. Biophys. Res. Commun.*, **37**, 313 (1969).
(46) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930. Here, $K_e = (K_p/K_s)(k_1k_2/k_{-1}k_{-2})$.
(47) (a) P. B. Sigler, D. M. Blow, B. W. Matthews, and R. Henderson, *J. Mol. Biol.*, **35**, 143 (1968); (b) D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature (London)*, **221**, 337 (1969). (Note: our discussion does not include the possible role of the carboxylate group of Asp-102.)
(48) P. W. Inward and W. P. Jencks, *J. Biol. Chem.*, **240**, 1986 (1965).
(49) W. F. Sager and P. C. Parks, *J. Amer. Chem. Soc.*, **85**, 2678 (1963); *Proc. Nat. Acad. Sci. U. S.*, **52**, 408 (1964).
(50) T. Inagami, A. Patchornik, and S. S. York, *J. Biochem. (Tokyo)*, **65**, 809 (1969).
(51) L. Parker and J. H. Wang, *J. Biol. Chem.*, **243**, 3729 (1968).
(52) M. Caplow, *J. Amer. Chem. Soc.*, **91**, 3639 (1969).
(53) J. H. Wang and L. Parker, *Proc. Nat. Acad. Sci. U. S.*, **58**, 2451 (1967).

Acylation of Chymotrypsin by Esters and Deacylation by Hydroxyl Compounds. Inward and Jencks[48] have also shown that the deacylation of furoyl chymotrypsin by alcohols in the pK_a range 12–16 is virtually independent of the pK_a. This requires that k_1/K_s for the hydrolysis of alkyl esters follows a β value of about -0.70. This is found for the hydrolysis of alkyl hippurates.[54,55] Aryl hippurates and mesyl phenylalaninates[56] show substantially lower β values of ~ -0.2. The ambiguity between mechanisms analogous to eq 39 and 40 for the deacylation of acyl chymotrypsins is resolved in favor of eq 40 as impossibly high rates would be necessary for eq 39.[57]

Papain. Less information is available as the determination of k_{-1} for various alcohols and amines does not give satisfactory linear free-energy relationships as specific binding sites are involved for these nucleophiles.[58,59] However, the hydrolysis of aryl hippurates exhibits a value of about -0.6 for k_1/K_s for phenyl substitution.[60] The deacylation of hippuryl papain by substituted phenols must, therefore, be almost independent of the pK_a when based on a rate law involving undissociated phenol.

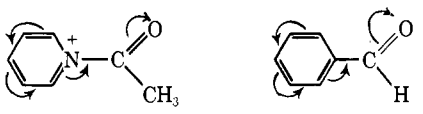
The acylation of papain by hippuryl anilides exhibits a β of $+0.3$–0.4 for k_1 based on aniline variation.[42] This is consistent with the rate-determining breakdown of a tetrahedral intermediate.

Although in nonenzymatic nucleophilic reactions thiols are not subject to general base catalyzed activation but react *via* the anion, proximity effects could cause the enzymatic reaction to be initiated by a general base mechanism rather than by a zwitterionic pair.[61]

Acknowledgment. We are grateful to Professor W. P. Jencks for his helpful comments on the manuscript, especially the suggestion of the inclusion of the Appendix.

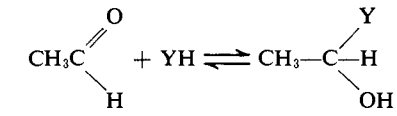
Appendix

Protonic and Tautomeric Equilibria of Amides and Imidates in Aqueous Solution at 25°. Our estimate for the pK_a of N protonation of amides (eq 23) based on the comparison of an acetylpyridinium ion to an acylated tertiary aliphatic amine ignores the resonance energy due to the interaction of the pyridine ring with the carbonyl group. This may be estimated by assuming that



(43)

it is similar to that in benzaldehyde. Pauling[62] quotes 4 kcal for this interaction. We arrive also at this figure by a comparison of the equilibrium constants for the addition of nucleophiles to acetaldehyde and benzaldehydes.



(44)

Using known equilibrium constants[63–66] and correcting for the polar contribution of the aromatic group by the equation of Greenzaid, *et al.*,[67] the resonance energy lost on formation of the tetrahedral adduct is calculated to be 4 ± 0.4 kcal.

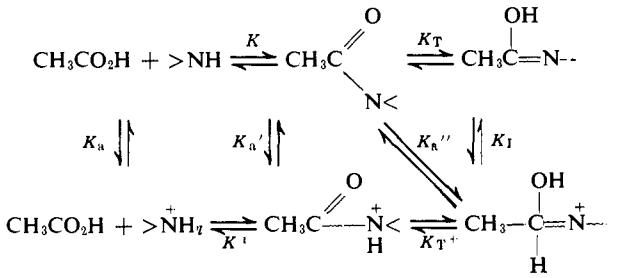
A further correction is required in that an N-protonated amide is not exactly equivalent to an acylated tertiary amine due mainly to different solvation energies. Trimethylamine is 0.9 unit less basic than methylamine or dimethylamine. These two compensating factors decrease the previously calculated pK_a values by 2 log units. Equation 45 is a better approximation than eq 23 for the N protonation of amides.

$$pK_a = -18.6 + 1.04pK_{a>NH} \qquad (45)$$

The resonance energy of amides may be calculated from this by consideration of the lowering of the pK_a of an amine on acetylation. The *inductive* effect of an acyl group lowers the pK_a of an amine by 5.4 units.[68,69] The residual lowering in eq 45, due to the loss of resonance on N protonation, is 13.2–0.04 $pK_{a>NH}$ units. For a typical amide of an amine of pK_a 10 the resonance energy is 17.5 kcal. This is in good agreement with the 21 kcal estimate of Pauling[71] and confirms the validity of the approximations.

Scheme IV represents the tautomeric and protonic

Scheme IV



equilibria of amides and imidates. We have measured and calculated values for K and K^+, K_a' and K_a's are well documented, and values are known for some K_a''.[32] K_I should be similar to the observed ionization constants of *O*-methyl or ethyl acetimidates, Hence, K_T and K_T^+ may be calculated. For example, when

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(55) R. M. Epand and I. B. Wilson, *J. Biol. Chem.*, **238**, 1718 (1963).
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(61) The argument in C. Lucas and A. Williams (*Biochemistry*, **8**, 5125 (1969)) against the participation of a zwitterionic mechanism is incorrect: the combination of a (k_c'/K_m) of 2.6×10^1 and a zwitterionic fraction of 10^{-4} in no way implies the existence of a rate constant in excess of diffusion control.
(62) L. Pauling, "The Nature of the Chemical Bond," 3rd ed, Cornell University Press, Ithaca, N. Y., 1960, p 198.
(63) E. Sander and W. P. Jencks, *J. Amer. Chem. Soc.*, **90**, 4377, 6154 (1968).
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(67) P. Greenzaid, Z. Lug, and D. Samuel, *ibid.*, **89**, 749 (1967).
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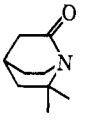

is decreased by 5.4 units.[70]
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>NH is methylamine, $K_I \sim 10^{-7.5}$,[72] $K_a'' = 10^{+0.46}$,[73] and K_a is calculated from eq 45 to be $10^{+7.6}$. Hence, K_T is 10^{-8} and K_T^+ is 10^{+7}.

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The equilibration of *O*-methylvalerolactim to *N*-methylvalerolactam at 130° in the liquid phase is accompanied by an enthalpy change of −17.4 kcal/mol or −14.1 ± 3.5 kcal/mol in the gas phase[74] in qualitative agreement with the above calculations.

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The Origin of the Heme Cotton Effects in Myoglobin and Hemoglobin[1-3]

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Abstract: The induced heme optical activity in heme proteins has been investigated theoretically. Myoglobin and hemoglobin were studied for their three-dimensional structures are known. The rotational strengths of the heme π–π^* transitions in these two proteins (Q, B (Soret), N, and L bands) were calculated according to Kirkwood's theory as extended by Tinoco, using atomic coordinates provided by Kendrew and Watson and by Perutz. We have examined several possible contributions, but the only one which can account for the observed Cotton effects is a coupled oscillator interaction between the heme transitions and allowed π–π^* transitions in nearby aromatic side chains. The calculated Soret rotational strengths are 0.3 and 0.1 DBM for myoglobin and hemoglobin, respectively, while the experimental values are 0.5 and 0.2 DBM. Calculations for other heme transitions—the Q, N, and L bands—lead to qualitative agreement with experiment in both proteins. The near degeneracy of the Soret transition has interesting consequences for the shape of the Soret CD band. The results indicate that the two Soret components are polarized toward the bridging methine carbons. Calculations on horse oxyhemoglobin show that the interactions of the heme in one subunit with aromatic groups in another subunit of the $\alpha_2\beta_2$ tetramer are not negligible. Heme Cotton effects of mutant hemoglobins and other heme proteins are discussed, based on the mechanism identified in myoglobin and hemoglobin.

One of the most interesting aspects of the optical activity of heme transitions in heme proteins is the induced nature of the Cotton effects. Heme proteins contain a prosthetic group which is a derivative of porphyrin. Because of its symmetry, the porphyrin alone is optically inactive. When it is bound to the protein, induced Cotton effects arise from the heme–protein interaction. In principle, the sign and magnitude of these effects should yield information about the nature of the heme binding site. The use of optical rotation to determine the helical content of proteins has been known for a long time. However, the study of protein conformation by the induced optical activity of prosthetic groups is a virtually unexplored field.[4]

Recently, Cotton effects associated with heme electronic transitions have been studied experimentally in a number of heme proteins, such as myoglobin,[5-9] hemoglobin,[5,10-13] cytochrome *c*,[11,14,15] and horseradish peroxidase.[4,8] A great variety in the signs, magnitudes, and shapes of the circular dichroism and optical rotatory dispersion curves has been observed for the heme transitions in these proteins. This indicates that the origin of the heme Cotton effects may be rather complicated. Several proposals have been made for the origin of these Cotton effects,[7,16] but no extensive study has been carried out.

In this paper we report theoretical calculations of the heme rotational strengths in myoglobin and hemoglobin. These molecules were chosen because their three-dimensional structures are known in considerable detail.[17,18] The origin of heme Cotton effects in other heme proteins will also be discussed based on the mechanism identified in hemoglobin and myoglobin.

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(1) Based in part on a thesis submitted by Ming-Chu Hsu in partial fulfillment of the requirements for the Degree of Doctor of Philosophy at the University of Illinois, 1970.
(2) Presented in part at the 158th National Meeting of the American Chemical Society, New York, N. Y., Sept 1969.
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