Solvent Effects in Reactions of Amino Groups in Amino Acids, Peptides, and Proteins with α,β-Unsaturated Compounds[1]

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Abstract: The reaction rates of amino groups in structurally different amino acids, peptides, and proteins with α,β-unsaturated compounds were studied in a medium consisting of 50% pH 8.4 buffer–50% dimethyl sulfoxide (DMSO) and were compared to analogous rates in an aqueous buffer. The presence of DMSO in the aqueous buffer resulted in a variable rate enhancement directly related to the pK_2 values of the amino groups. A plot of the logarithm of the ratio of second-order rate constants determined in the mixed solvent system to that in the aqueous buffer *vs.* pK_2 is linear. The straight line is described by the relationship $R = 0.855pK_2 - 6.505$. Rates in the mixed solvent system are more sensitive to changes in basicities of amino groups than corresponding rates determined in aqueous buffers. Additional studies were carried out on the influence of several variables on rates. The results are rationalized in terms of participation of aprotic solvents in acid–base equilibria, stabilization of ground and transition states, and hydrogen-bonding interactions. The configuration and conformation of a protein appear to be significant in governing relative reactivities of its amino groups.

Dipolar aprotic solvents, like dimethyl sulfoxide (DMSO), are known to accelerate a number of organic reactions, presumably because they are able to solvate cations preferentially and thus free the anion from the destabilizing influence of a positive charge and ion-pair aggregation.[3–6] Such a mechanism of catalysis by dipolar solvents explains the large rate-enhancing effect of these solvents in nucleophilic displacement reactions in which negatively charged nucleophiles participate. Although amino acid anions of structure NH_2RCOO^- are negatively charged nucleophiles, the negative charge is not located on the amino group that is involved in nucleophilic displacement and addition reactions. It was therefore of theoretical interest to establish the influence of nonaqueous solvents on reaction rates of these unique nucleophilic species.

The reaction selected for this study is a Michael-type nucleophilic addition as illustrated by glycine and acrylonitrile

$$\underset{\text{I}}{^-OOCCH_2\overset{+}{N}H_3} + B \underset{}{\overset{K_2}{\rightleftharpoons}} \underset{\text{II}}{^-OOCCH_2NH_2} + BH^+ \quad (1)$$

$$\underset{\text{II}}{^-OOCCH_2NH_2} + \underset{\text{III}}{CH_2{=}CHCN} \longrightarrow$$

$$^-OOCCH_2\overset{+}{N}H_2{-}CH_2{-}\bar{C}HCN \quad (2)$$

$$\text{IV}$$

$$\downarrow$$

$$^-OOCCH_2NHCH_2CH_2CN$$

$$\text{V}$$

A base removes a proton from zwitterion I to give the anion II, which then reacts in the rate-determining step with acrylonitrile III.

In previous communications in this series, studies are described on the nature of parameters that govern reactivities of functional groups present in proteins with α,β-unsaturated compounds[7–11] and with ninhydrin.[12] Linear free-energy relationships were developed which correlate the observed results in terms of polar, steric, and nucleophilic parameters associated with each of the reactants.

The latest study was designed to determine the relative influence of nonaqueous solvents on reaction rates of amino groups with α,β-unsaturated compounds to delineate the mechanism of interaction of these solvents with ground and transition states. Reaction rates of amino groups in structurally different amino acids, peptides, and proteins were studied in media consisting of mixtures of nonaqueous solvents and aqueous buffers. The observed rates were compared to analogous rates determined in the aqueous buffers.

To gain some insight into the mechanism of the solvent effects at a molecular level, a number of variables were investigated. These include steric environment and basicities of the amino groups, nature of the vinyl compounds, nature of the solvent, pH of the aqueous buffer, and concentration of dimethyl sulfoxide. The observed results are explained in terms of solvation mechanisms responsible for the increased basicities and nucleophilicities of the amino groups in the ground state and for better stabilization of the transition states. Evidence indicates that dimethyl sulfoxide affects hydrogen-bonding interactions which influence rates.

Results and Discussion

Effect of Basicities of Amino Groups on Rates. Rates of reaction were followed by the ninhydrin colorimetric procedure previously described,[7] which measures the

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Table I. Rates of Reaction with Acrylonitrile at 30° as a Function of pK_2 Values of Amino Groups (units for all rate constants are in l./mole sec)

No.	Amino compd	pK_2[a]	k_A[-a] × 10^4	k_2[a] × 10^4, pH 8.4 (A)	k_2 × 10^4, 50% pH 8.4–50% DMSO (B)	Ratio B/A
1	Tetraglycine	7.63	9.81	8.32	11.5	1.39
2	Diglycine	8.04	13.7	9.54	15.5	1.62
3	Glycine	9.47	50.0	3.92	154.0	39.3
4	β-Alanine	10.06	89.2	1.91	186.0	97.5
5	γ-Aminobutyric acid	10.41[b]		1.30[c]	232.0	178.5
6	ε-Aminocaproic acid	10.62	203.0	1.21	253.0	209.1
7	L-Alanylglycine	8.07[b]		5.03[c]	6.65	1.32
8	DL-Phenylalanine	9.00	17.6	3.51[c]	49.8	14.2
9	L-Tyrosine	9.00[b]		3.51	66.2	18.8
10	DL-Methionine	9.08	17.6	3.04	66.3	21.8
11	L-Leucine	9.43[b]		2.57[c]	90.0	35.0
12	DL-α-Alanine	9.57	35.3	2.23	81.7	36.6
13	DL-Norleucine	9.63	34.9	2.05	87.0	42.4
14	DL-α-Phenyl-α-alanine	9.12	1.05	0.167	5.35	32.0
15	DL-α-Methylmethionine	9.45	1.56	0.128	6.63	51.8
16	DL-Isovaline	9.98	2.79	0.0715	10.0	140.0
17	α-Aminoisobutyric acid	10.02	3.17	0.0742	13.8	186.1
18	1-Aminocyclopentane-1-carboxylic acid	10.06	4.51	0.0966	17.4	180.1

[a] Values from ref 7 except as indicated. [b] Values from D. D. Perrin "Dissociation Constants of Organic Bases in Aqueous Solutions," Butterworth and Co., Ltd., London 1965. [c] Predicted rate constants calculated by means of eq 5 and 7 in ref 7.

amount of primary amino compound in the reaction mixture and gives negligible color with alkylated amino compounds such as V. The pseudo-first-order kinetic procedure was used to obtain the second-order rate constants k_2 (see Experimental Section).

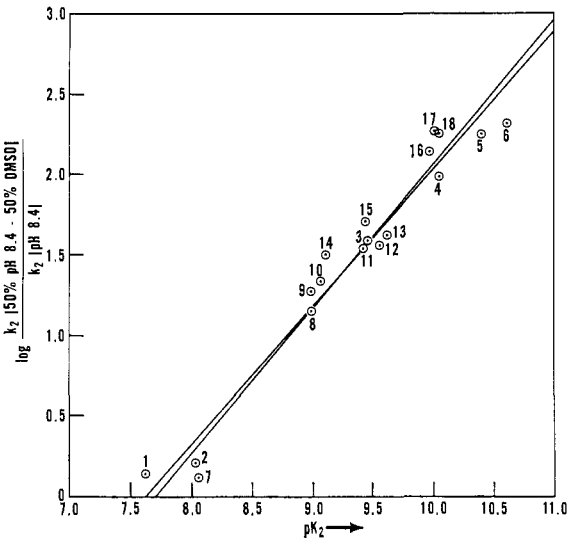


Figure 1. Plot of ratios of second-order rate constants (k_2 × 10^4 in l./mole sec) for the reaction of amino groups in amino acids and peptides with acrylonitrile at 30°. Numbers correspond to compounds listed in Table I.

The standard medium consisted of equal volumes of DMSO and pH 8.4 borate buffer. Rates at pH 8.4 were previously determined at an ionic strength of 1.2, but it was necessary to reduce the ionic strength of the buffer in the mixture to 0.15, because addition of DMSO to buffers of higher ionic strength caused precipitation of KCl. Ionic strength has only a slight effect on reactions of amino and thiol groups with acrylonitrile.[8,9]

Table I summarizes rate data for the reaction of three structurally different series of amino acids and peptides with acrylonitrile. In the first series (1–6), the amino groups are attached to primary carbon atoms; in the second (7–13), to secondary; and in the third (14–18), to tertiary. The amino groups are in a similar steric environment within each series. Second-order rate constants (k_2) in a medium consisting of 50% pH 8.4 buffer–50% DMSO are compared to analogous rate constants determined in the pH 8.4 buffer. The ratio of rates shown in the last column is taken as a measure of the rate-enhancing effect of the added DMSO.

In the pH 8.4 buffer, rates decrease with increasing pK_2 values for each series, because the greater the pK_2 the lower the reactive amino acid anion concentration. Observed rates in the 50% pH 8.4–50% DMSO medium, however, parallel the pK_2 values of the amino groups. Indeed, a plot of the logarithm of the ratio of rates for all 18 compounds against pK_2 values is nearly linear (Figure 1). The straight lines in Figure 1 were drawn by standard least-square fit methods. The required calculations and plotting were performed with an IBM 1130 computer system equipped with an IBM 1627 plotter. The sum of deviations squared in the x direction was taken as a minimum for the steeper line in Figure 1, and the sum of deviations squared in the y direction was taken as a minimum for the other line. The flat line is described by the equation

$$\log \text{ ratio of rates} = R = 0.855pK_2 - 6.505 \quad (3)$$

with a standard error of estimate in R of 0.1487. The relationship for R may be used to calculate rate ratios for any amino acid or peptide whose pK_2 is known. For example, the predicted rate ratio for a compound with a pK_2 value of 10 would be 111 ± 14.

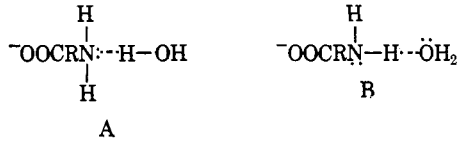
The variable rate enhancement of DMSO can be explained in terms of relative participation of H_2O and DMSO in acid–base equilibria and hydrogen-bonding interactions in the ground and transition states.

The pK_2 of an amino acid is a constant that measures not only the concentration of the amino acid anion at any given pH, but also the inherent basicity of an amino group. Observed rates for a sterically similar series

of amino acids and peptides decrease with increasing pK_2 values. The dependence of reaction rates on differences in inherent basicities of amino groups can be deduced by comparing second-order anion rate constants (k_A-) as a function of pK_2 values of amino groups. These rate constants either may be secured directly by carrying out rate studies at a pH about two units above the pK_2 values or may be calculated from rates at any pH by means of the previously derived[7] formula k_A- = $k_2(1 + (H^+)/K_2)$. The anion rate constants which measure maximum rates in aqueous solution are a direct function of the basicities of the amino groups and are pH independent.

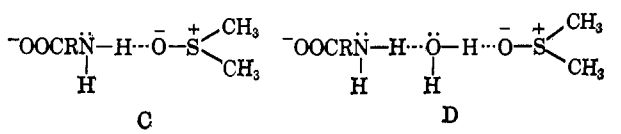
Since the observed rate constants in the 50% pH 8.4–50% DMSO medium are also a direct function of the pK_2 values, apparently the added DMSO causes complete ionization of the ammonium group. Some support for this hypothesis comes from the observation that the pH of solutions of all amino compounds in the 50% pH 8.4–50% DMSO medium was between 10.5 and 11.0. Although the validity of such pH readings in mixed aqueous and nonaqueous solvent media could be questioned, all compounds with pK_2 values of 9 or less would be completely ionized in the cited pH range. The effect of DMSO on the ionization equilibrium, however, could not be its only influence for two reasons. First, the observed k_2 values in the mixed medium are greater than the k_A- values by a factor ranging from 1.2 to 5.0 (Table I). This difference signifies that the inherent nucleophilic reactivities of the amino groups are greater in the 50% pH 8.4–50% DMSO solvent medium than the maximum attainable nucleophilicities in aqueous buffers. Second, the slope in Figure 1 (0.855) is double the average slope (0.427) from plots of log k_A- *vs.* pK_2 values. Rates in the mixed solvent system are more sensitive to changes in pK_2 values than corresponding rates determined in aqueous buffers.

The strong hydrogen-bond donor ability of water will result not only in effective solvation of anions but also in formation of a hydrogen bond with the amino group of the conjugate base of an amino acid, as in A. The



A B

electron density on nitrogen is thereby effectively decreased and, consequently, its nucleophilicity is lowered. This process also counteracts hydrogen-bond donation by the amine to water, as in B, which would enhance nucleophilicity of the nitrogen. In DMSO, only the B-type hydrogen bonding is possible since DMSO is a strong hydrogen-bond acceptor only. The DMSO could therefore increase the nucleophilicity of the conjugate base of an amino acid by (1) decreased solvation of the negative charge in A or B or (2) hydrogen-bond formation between DMSO oxygen and an NH_2 hydro-



C D

gen, *i.e.*, similar to B or as in C and D. Because of poorer solvation of the negative carboxyl group in the mixed solvent, C and D ground states would be of higher energy than A and B.

Similar hydrogen-bonding effects may occur in the transition state (eq 2), but because of more diffuse charge and steric bulk, there would likely be less difference in solvation between protic or aqueous solvents and aprotic–dipolar solvents. Since DMSO is more selective in its solvation of the transition state, due to selective solvation of the positive charge, one would expect a more positive ΔS^* for the formation of the transition state in the mixed solvent system than in the aqueous buffer (bulk or steric effect), provided solvation by DMSO does not produce a more ordered system than corresponding solvation by H_2O (ordering or restrictive effect).

Reaction Rates as a Function of pH of the Buffer. The relationship between buffer pH and reaction rates was established by determining the rate constants for reaction of diglycine with acrylonitrile as a function of buffer pH. Table II shows that the rate increases with pH and becomes constant above pH 8.4. Interestingly, the measured pH values of the reaction media increase in a parallel fashion with the buffer pH.

Table II. Reaction Rate of Diglycine with Acrylonitrile as a Function of pH of the Buffer at 30°

Reaction medium	$k_2 \times 10^3$, l./mole sec	pH[a]
50% H_2O–50% DMSO	0.04	5.5
50% pH 7.5[b]–50% DMSO	0.27	7.6
50% pH 7.9[c]–50% DMSO	1.3	9.3
50% pH 8.4[c]–50% DMSO	1.5	10.6
50% pH 9.0[c]–50% DMSO	1.5	11.8
50% pH 11.0[c]–50% DMSO	1.5	>13.0

[a] Average of measured initial and final pH values of reaction mixture. [b] Tris buffer. [c] Borate buffer.

With a pK_2 value of 8.04, diglycine would be incompletely ionized in a pH 8.4 medium. Since a further increase in pH of the buffer does not result in a corresponding increase in rate in the mixed medium but does cause an increase in rates in the aqueous buffer media, evidently added DMSO favors ionization of the zwitterion to the anion.

Effect of DMSO Concentration on Rates. The effect of varying DMSO concentrations on rates was studied with two peptides and one amino acid (Table III).

Table III. Second-Order Rate Constants ($k_2 \times 10^4$ in l./mole sec) for Reaction of Amino Groups with Acrylonitrile at 30° as a Function of DMSO Concentration

Compd	pH 8.4	20% DMSO	50% DMSO	80% DMSO
Diglycine	9.5	14.4	15.5	2.3
L-Alanylglycine	5.0	6.0	6.3	0.93
α-Aminoisobutyric	0.074	1.32	13.8	21.5

With the peptides, rates pass through a maximum and then decrease with increasing DMSO concentration, whereas with α-aminoisobutyric acid, the rate increases with DMSO concentration. Peptide bonds appear to be responsible for decreased reactivities of the two dipeptides at the higher DMSO concentration, but no

Table IV. Second-Order Rate Constants ($k_2 \times 10^4$ in l./mole sec) for Reaction of Amino Groups with α,β-Unsaturated Compounds at 30°

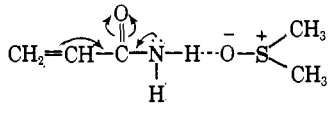
Compd	k_2,[a] pH 8.4 (A) Diglycine	Glycine	k_2, 50% pH 8.4–50% DMSO (B) Diglycine	Glycine	Ratio B/A Diglycine	Glycine
CH_2=$CHCONH_2$	1.3	0.49	0.38	0.41	0.30	0.84
CH_2=$CHCON(CH_3)_2$	0.21	0.072	0.20	0.29	0.95	4.0
CH_2=$CHCN$	9.5	3.9	15.5	155	1.62	39.3
CH_2=$CHSO_2CH_3$	62.7		103.0		1.65	

[a] Predicted rates calculated from data in ref 9 by means of eq 1.

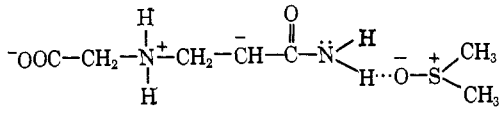
obvious explanation can be offered to rationalize this solvent effect.

Reliable data could not be obtained in 100% DMSO medium because of solubility problems.

Effect of Structure of Vinyl Compounds on Rates. Direct evidence which implicates DMSO in hydrogen-bonding interactions comes from a comparison of observed relative reactivities of four vinyl compounds (Table IV). The striking result is that addition of DMSO causes a retardation in the rate with acrylamide. This unexpected behavior must be due to the amide group in acrylamide which may participate in intra- or intermolecular hydrogen bonding with various charged species in the reaction mixture. A possible mechanism for such interactions between the strong dipole of the sulfoxide group in DMSO and the amide group of acrylamide is depicted in E. The indicated hydrogen



E



F

bonding in E would increase the electron density on nitrogen. The resulting greater conjugative interaction (a) of the lone electron pair on nitrogen competes with the conjugative polarization (b) of the π electrons of the carbon–carbon double bond by the carbonyl group. The net effect would be an increased electron density at the terminal carbon atom of acrylamide and a decreased electrophilic reactivity for this vinyl compound. Similar influences operate in the transition state F.

The observed reaction rates for N,N-dimethylacrylamide support this hypothesis. Since the hydrogen-bonding interactions described are not possible, the ratios of reaction rates in the mixed solvent system to those in the aqueous buffer (B/A ratios in Table IV) are three to four times greater for N,N-dimethylacrylamide than the corresponding values for acrylamide.

It should also be pointed out that two additional factors contribute to the electrophilic reactivity of N,N-dimethylacrylamide. These are the steric and inductive influences of the two methyl groups. The electron-donating inductive effect probably enhances the conjugative interaction of the unpaired electrons on nitrogen with the carbonyl group in N,N-dimethylacrylamide. The two parameters are undoubtedly also subject to solvent effects.

Effect of Nonaqueous Solvents on Rates. The effect of varying the nature of the nonaqueous solvents on rates was studied with glycine and diglycine (Table V).

Table V. Second-Order Constants ($k_2 \times 10^4$ in l./mole sec) for the Reaction of Amino Groups with Acrylonitrile at 30° as a Function of Nonaqueous Solvent

Reaction medium	Glycine	Diglycine
pH 8.4	3.9	9.5
50% pH 8.4–50% methanol	7.3	8.2
50% pH 8.4–50% acetonitrile	18.0	3.5
50% pH 8.4–50% dimethylformamide	93.0	9.0
50% pH 8.4–50% dimethylacetamide	93.0	7.5
50% pH 8.4–50% dimethyl sulfoxide	155.0	15.5

The data in Table V indicate a lack of solvent sensitivity of diglycine as compared to glycine. Interestingly, rates for both glycine and diglycine are similar in the presence of methanol, a nonaqueous protic solvent.

Reactivities of Protein Amino Groups. The progress of reaction of amino groups with acrylonitrile in three proteins at pH 8.4 and in a 50% pH 8.4–50% DMSO medium is illustrated in Figures 2 and 3, respectively. Since amino groups react at different rates in the three proteins and since the expected straight-line pseudo-first-order plots do not generally occur, reactivities change within the same protein. The curvature of the plots is more pronounced in the mixed solvent medium than in the aqueous buffer.

Evidently, readily accessible amino groups on the surface of the protein molecule react at faster rates than analogous amino groups which are partially or fully hidden by folding and helicity of the protein chains.

Furthermore, since inherent basicities of amino groups are a function of their macro- and microscopic environment, acid–base equilibria for amino groups on the same protein chain must also vary. The conformation of the protein and the inherent microscopic and macroscopic basicities of its amino groups in a given reaction medium are probably the dominant factors that govern their nucleophilic reactivities.

Experimental Section

Source of Materials. All amino acids and peptides were the best commercial grades available.[13] Polylysine hydrobromide (mol wt 52,000) was purchased from Pilot; bovine albumin, crystallized, and lysozyme, three times crystallized, from Pentex; acrylamide, acrylonitrile, acetonitrile, dimethylformamide, and methanol from Matheson; N,N-dimethylacrylamide from K & K Laboratories; dimethylacetamide from Eastman; and dimethyl sulfoxide from Baker. Acrylonitrile, methyl vinyl sulfone, and dimethylacetamide were distilled before use. Dimethyl sulfoxide was dried over calcium hydride for 24 hr and then distilled under vacuum. Acrylamide was purified by sublimation. The other solvents were spectroquality grade and were used directly.

(13) The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

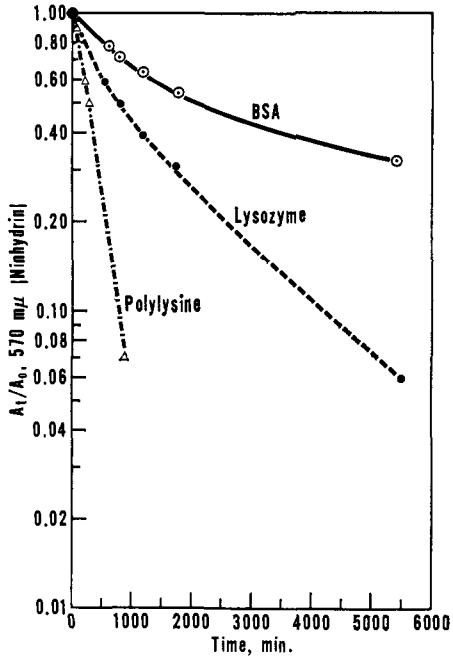


Figure 2. Plot of log A_t/A_0 *vs.* time for reaction of proteins (0.005 *M* in NH_2 groups) with acrylonitrile (0.0825 *M*) in pH 8.4 buffer medium (μ = 0.15) at 30°. BSA stands for bovine serum albumin.

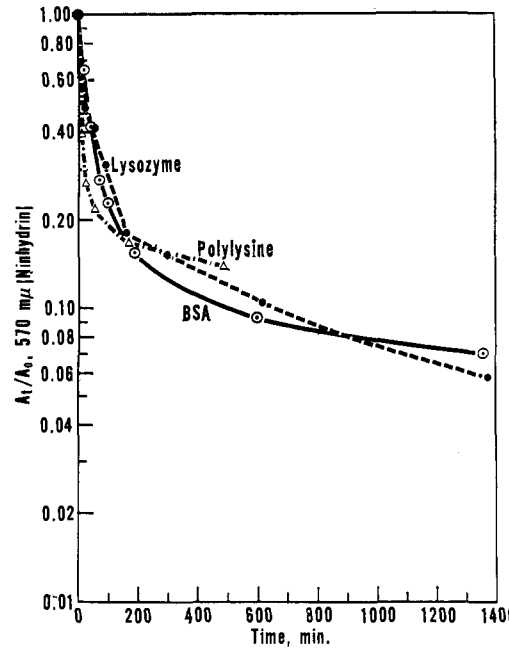


Figure 3. Plot of log A_t/A_0 *vs.* time for reaction of proteins (0.005 *M* in NH_2 groups) with acrylonitrile (0.0825 *M*) in 50% pH 8.4 buffer–50% DMSO at 30°. BSA stands for bovine serum albumin.

Kinetic Measurements. The general procedure has been described.[7] The concentration of the amino compounds ranged between 0.0025 and 0.01 *M* and that of the vinyl compound in the final mixture was 15 to 20 times in excess over the amino component. Protein concentrations were calculated on the basis of 0.005 *M* of amino groups. For polylysine, this calculation corresponded to 1.045 g/l. for lysozyme to 12.9 g/l. based on seven amino groups per mole and a molecular weight of 14,200, and for bovine serum albumin to 5.6 g/l., based on 58 amino groups per mole and a molecular weight of 65,000. The final acrylonitrile concentration in the protein solutions was 0.0825 *M*.

The following is a typical kinetic procedure for the reaction of diglycine with acrylonitrile. The reaction medium was prepared by mixing equal volumes of DMSO and pH 8.4 borate buffer (ionic strength 0.15). The resulting solution was equilibrated in a 30° water bath and used to dissolve 33 mg of diglycine in a 50-ml volumetric flask (solution A). Ten milliliters of this solution was removed by means of a pipet (solution C).

The pH of solution A was 10.6 as read on a TT1C titrator with a titrigraph (Radiometer-Copenhagen). To solution A and to 40 ml of a blank solution consisting of the solvent mixture (solution B) was added 0.15 ml of redistilled acrylonitrile. The tightly stoppered flasks were shaken vigorously and placed in the 30° bath together with one containing solution D, which consisted of the solvent mixture alone. Periodically, 1-ml aliquots were removed from solutions A, B, C, and D and diluted to 25 ml with water. The ninhydrin color reaction, according to the procedure of Stein and Moore,[14] was carried out in triplicate with three 1-ml aliquots of these dilutions. The fraction of amino components remaining unreacted was calculated from the formula $A_t/A_0 = A_A - (A_B/A_C) - A_D$ = fraction of primary amino compound left in solution A, where A_A, A_B, A_C, and A_D are the absorbances of solutions, A, B, C, and D, and where A_t and A_0 are the corrected absorbances at time t and zero, respectively. The reaction was left running for about four half-lives and the final pH reading of solution A was 10.5.

A plot was made of log A_t/A_0 *vs.* time. The half-life ($t_{1/2}$) was read from the linear plot, and the pseudo-first-order rate constant (k_1) and second-order rate constant (k_2) were calculated by the formulas $k_1 = 0.693/t_{1/2}$ and $k_2 = k_1$/concentration of vinyl compound.

The precision of the rate determination is illustrated with the following three values for the reaction of diglycine with acrylonitrile in a 50% pH 8.4–50% DMSO medium: $k_2 \times 10^4$ = 1.64, 1.47, and 1.54. Average and standard deviation = 1.55 ± 0.08.

Acknowledgment. I thank Mr. J. O. Ernst for the regression analysis computer program.

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