chloride yielded epiibogamine which was further purified by sublimation *in vacuo* (38 mg.), m.p. 163–165° (lit.[26] m.p. 162–164°).

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Reactions in Frozen Systems. II.[1] Enhanced Hydroxylaminolysis of Simple Amides

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Contribution from the Research Division, Wyeth Laboratories, Radnor, Pennsylvania. *Received March 15, 1965*

Hydroxylaminolysis of the amide bonds of formamide, acetamide, propionamide, glutamine, asparagine, and 2,5-diketopiperazine was studied in ice and in liquid water at several pH values. For acetamide, propionamide, glutamine, asparagine, and 2,5-diketopiperazine, hydroxylaminolysis rates in ice (−18°) exceeded the rates in water (0°) at pH 7 and 8. With glutamine and 2,5-diketopiperazine, the rates at −18° exceeded the rates at 22° at these pH levels. The reaction rate with 2,5-diketopiperazine in −18° frozen solutions exceeded the rate in −11° frozen solutions at pH 6, 7, and 8. The ratios k_1(pH 7)/k_1(pH 6) and k_1(pH 8)/k_1(pH 6) were less than 1.0 in each liquid system but greater than 1.0 in the frozen systems. These results indicate that the phenomenon of rate enhancement in frozen solutions is not limited to ordinarily facile reactions, and the observed differences between liquid and solid systems are inconsistent with a concentration effect in the solids. In addition to mechanisms previously proposed, it is suggested that the dielectric properties of ice may facilitate a concerted attack on the substrate by favoring association of nucleophile molecules.

Introduction

Enhanced rates in ice for the catalytic hydrolysis of the penicillin β-lactam were reported earlier from this laboratory.[1] The evidence did not support the explanation of increased reactant concentrations, and it was suggested that in ice the dielectric properties, the high proton mobility, and the imposition of a favorable orientation of substrate and catalyst might explain the phenomenon. Since this report there have been at least five others showing reaction rate increases in ice.[2–6] Because this is a new area of investigation, it is important to learn whether the reactions which are facilitated in ice are limited to those which occur readily in water. Therefore we extended the study to hydroxylaminolysis of some of the most stable carboxylic acid derivatives, the primary amides and the peptides.

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Experimental Section

The chemicals used were reagent grade or chromatographically pure. Propionamide was synthesized from the anhydride and recrystallized from benzene. Two lots of hydroxylamine hydrochloride were used: Baker analyzed reagent, assaying 99.5%, was used directly, and Fisher reagent grade, assaying at least 96.5%, was recrystallized from aqueous ethanol. Asparagine was used in the L-form and glutamine in the racemic form.

The reaction mixture contained 0.005, 0.01, or 0.02 *M* substrate, 0.6 *M* hydroxylamine hydrochloride, and sufficient sodium hydroxide to provide a self-buffering system of the desired pH. Solutions at pH 6 and 7 were prepared both with and without the addition of sufficient sodium chloride to equal that finally present in the pH 8 solutions (0.6 *M*). The solutions were distributed into at least six test tubes, stoppered, and set at the appropriate temperatures. For studies at −18°, the solutions were first frozen rapidly in Dry Ice–acetone and then placed in a −18° deep freeze. For −11, 0, and 22° the locations were a thermostated cold room, refrigerator, and laboratory. All samples at 0° remained liquid. Frozen samples were thawed by immersion in a room temperature water bath and then mixed again by holding against a Vortex mixer. The pH after incubation was measured on a Beckman expanded scale pH meter.

For assay, 3-ml. samples were mixed successively with 3 ml. of 0.935 *M* HCl and 1 ml. of 15% ferric ammonium sulfate in 1 *N* H_2SO_4, giving the iron complex whose absorbance was read on a Klett–Summerson spectrophotometer with a 540 mμ filter. In the presence of a large excess of hydroxylamine almost all of the reactions followed clean first-order kinetics for at least two half-lives, and rates were calculated from plots of the first-order equation, $\log (a - x) = -(k_1/2.303)t + \log a$. Determination of the concentration, *a*, of four of the amides was based upon quantitative alkaline hydroxylaminolysis methods.[7,8] In the procedure adopted, 1-ml. samples were incubated with 1 ml. of 1.8 *M* hydroxylamine hydrochloride and 1 ml. of 3.5 *M* sodium hydroxide. Time, temperature, and absorbance for 0.01 *M* amide (from the plot) are as follows: formamide, 6 min., 22°, 1.890; acetamide,

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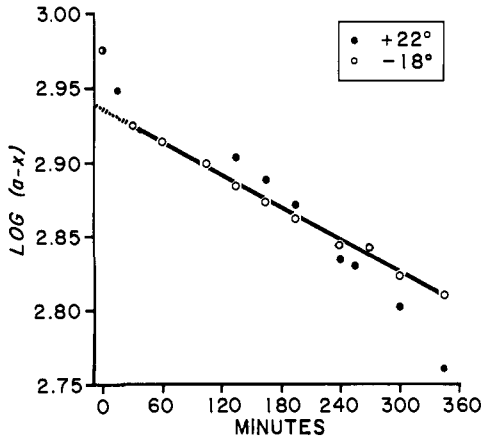

Figure 1. First-order rate plots for hydroxylaminolysis of formamide at pH 8.

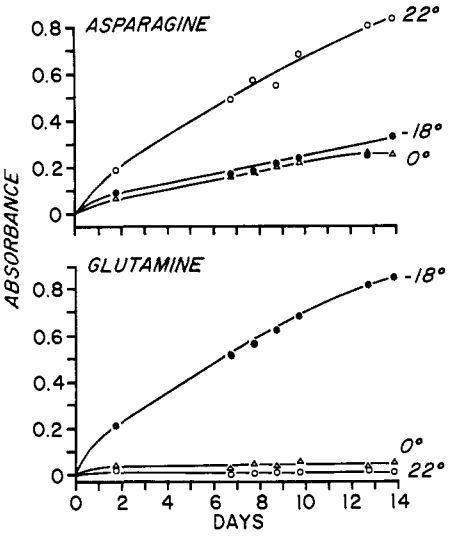

Figure 2. Hydroxylaminolysis of asparagine and glutamine at pH 8.

180 min., 22°, 3.800; propionamide, 240 min., 22°, 2.650; L-asparagine, 180 min., 60°, 1.650. Values of a for 2,5-diketopiperazine and glutamine were taken from kinetic runs, in which final hydroxamic acid color yields (absorbances of 1.160 and 0.688, respectively, for 0.01 M amide) exceeded those derived from alkaline hydroxylaminolysis.

Results

Final pH values in a given experiment were the same regardless of whether the solutions had been liquid or frozen. In solutions initially at pH 8.0, the pH dropped an average of 0.16 unit (90 determinations with 0.01 M substrate), and solutions initially at pH 6.0 and 7.0 usually changed by less than 0.1 pH unit during the period of the run (2–3 half-lives). This agrees with the finding of Jencks and Gilchrist[9] that 0.1–1.5 M hydroxylamine hydrochloride buffered adequately between pH 5 and 8 with 0.005 M amide. In addition to hydroxylaminolysis, a factor capable of releasing ammonia and influencing the pH of these systems is hydrolysis, but hydrolysis is insignificant with the more labile butyl thiolacetate at pH 5.3[10] and with formamide at pH 12–13.[9]

Good agreement was found when first-order rate constants were determined at different concentrations of substrate. For example, at −18° and pH 8, 0.005 and 0.01 M substrate gave these constants [$10^5 \cdot k_{obsd}$ (min.$^{-1}$)]: glutamine, 14.5 and 14.4; asparagine, 1.08 and 1.06; propionamide, 2.62 and 2.62; acetamide, 2.32 and 2.53.

Formamide. Hydroxylaminolysis of formamide followed first-order kinetics at pH 6 in both 22° and −18° systems but not at pH 8 in the 22° system (Figure 1). In frozen systems formamide was more reactive than any other amide studied except 2,5-diketopiperazine. At −18° the rate at pH 8 was more than six times that at pH 6.

Acetamide and Propionamide. These two amides reacted with hydroxylamine at similar rates under various conditions. The −18° rates exceeded the 0° rates at pH 7 and 8 but not at pH 6 (Table I). At pH 6, the k_1 values at −18° were lower than those at 22° by factors of 12 (acetamide) and 10 (propionamide), but at pH 8, both of the −18° rate constants were about 81% of the +22° rate constants.

Table I. Hydroxylaminolysis of Acetamide and Propionamide at 0 and −18°[a]

Substrate	Temp., °C.	$10^5 k_{obsd}$, min.$^{-1}$ pH 6	pH 7	pH 8
Acetamide	0	3.49	1.78	1.04
	−18	1.87	2.46	2.53
Propionamide	0	3.58	1.59	0.94
	−18	2.46	3.13	2.62

[a] Ionic strengths adjusted with sodium chloride.

Asparagine. As with acetamide and propionamide, the −18° rates were higher than the 0° rates at pH 7 and 8 but not at pH 6 (Table II). This was observed both with and without the addition of sodium chloride to the systems at pH 6 and 7.

Table II. Hydroxylaminolysis of Asparagine at 0 and −18°

Temp., °C.	$10^5 k_{obsd}$, min.$^{-1}$ pH 6	pH 7	pH 8
	Ionic strength adjusted with NaCl		
0	1.03	0.74	0.83
−18	0.31	0.99	1.06
	No NaCl added		
0	0.60	0.92	0.83
−18	0.14	1.32	1.06

Glutamine. The rates in the frozen solutions relative to those in the liquid solutions shifted with pH (Table III). At pH 7 and 8, with no addition of sodium chloride, the reactions were faster at −18° than at +22°. In the system with ionic strength adjusted, the rate at 0° decreased on changing from pH 6 to pH 7, while that at −18° increased with this pH rise. The rate at pH 8 was about zero at 22° (Table II, Figure 2); at 0° it was also very low and did not give a satisfactory first-order plot. Figure 2 shows the marked difference between glutamine and asparagine with respect to relative 22 and −18° rates at pH 8.

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Table III. Hydroxylaminolysis of Glutamine at 22, 0, and −18°

Temp., °C.	$10^5 k_{obsd}$, min.$^{-1}$ pH 6	pH 7	pH 8
Ionic strength adjusted with NaCl			
0	8.72	2.50	...
−18	9.97	17.1	14.4
No NaCl added			
22	29.4	12.7	0
0	9.20	5.83	...
−18	4.03	16.9	14.4

2,5-Diketopiperazine. This amide illustrates well the different hydroxylaminolysis rates in liquid and frozen aqueous systems (Figure 3, Table IV). At each pH

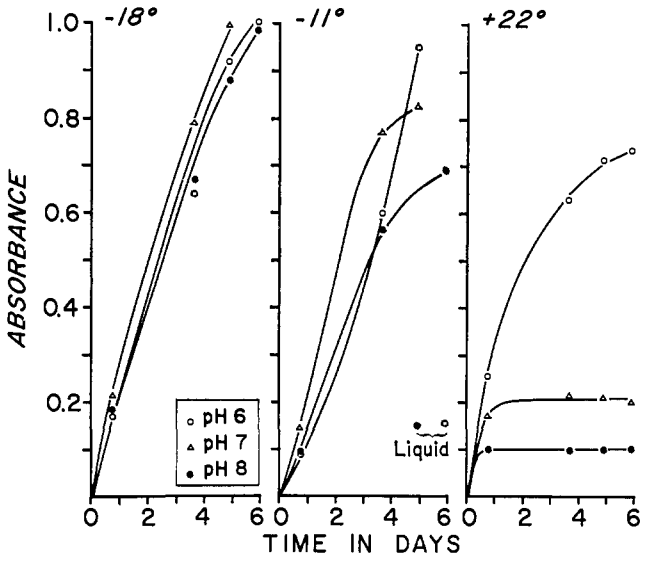

Figure 3. Hydroxylaminolysis of 2,5-diketopiperazine.

value the rate at −18° exceeded that at −11°. Each system at −18°, except that at pH 6 to which salt was added, reacted more rapidly than the corresponding liquid at 0°. At 0° the rate was faster at pH 6 than at pH 7 or 8. At −11 and −18° the rate was highest at pH 7. At −11° one sample at pH 6 and one at pH 8 failed to freeze, and, as shown in Figure 3, little reaction took place.

Table IV. Hydroxylaminolysis of 2,5-Diketopiperazine at 0, −11, and −18°

Temp., °C.	$10^5 k_{obsd}$, min.$^{-1}$ pH 6[a]	pH 6	pH 7	pH 8
0	4.36	5.18	3.84	2.07
−11	1.73	12.5[b]	17.7	10.2
−18	3.69	14.8	21.3	16.1

[a] Ionic strengths adjusted with sodium chloride. [b] The semilogarithmic plot was linear for only about one half-life, on which the calculation was based.

The marked fall in rate at 22° and pH 7 and 8 is possibly attributable to catalysis of polymerization to various polyglycines. Glycylglycine did not undergo measurable hydroxylaminolysis when studied at pH 8 during a period of 3 days at 22° or −18°.

The Effect of Sodium Chloride. The addition of sodium chloride (0.3 *M* at pH 6; 0.1 *M* at pH 7) influenced the reactions at −18° in several ways (Tables I–IV). Rate decreases of more than 50% were observed with acetamide and 2,5-diketopiperazine at pH 6. Rate increases of more than 50% were observed with propionamide, asparagine, and glutamine at pH 6, where little or no change was found at pH 7. The rate of the glutamine reaction at 0° and pH 6 changed very little with salt addition, but at 0° and pH 7 it was lowered by 57% (Table III).

Discussion

Previous studies on reactions of the carboxylic acid derivatives in ice have dealt with labile groups such as the fused β-lactam,[1] the acid anhydride,[4] lactone,[4,5] and nitrophenyl ester.[4] The amide link is exceptionally stable, probably owing to the resonance which results in a masking of the carbonyl function. The present study with amides shows that enhanced reaction rates in ice are not confined to reactions known to be facile under other conditions.

In the pH range 6–8, the relation between hydroxylaminolysis rate and physical state of the solution shifted on going to higher pH levels. The ratios of rate constants at pH 7 or 8 to that at pH 6 were less than 1.0 in each liquid system but greater than 1.0 in the frozen systems. The reaction between hydroxylamine and amides can be catalyzed by hydroxylammonium ion, by the free base, and by various buffers.[9] Change in the pH–rate relation suggests a shift in ice toward increased relative importance of base catalysis, rather than a reaction proceeding by the same mechanism at a higher rate governed by higher reactant concentrations. This interpretation of the kinetic data is similar to that of Bruice and Butler,[5] who found that the morpholinolysis of thiolactones changes from third order in liquid water to an unassisted nucleophilic displacement in frozen systems, and it supports the conclusions of Grant, *et al.*,[1] on the failure of a concentration effect to explain the imidazole- and histidine-catalyzed hydrolyses of penicillins in ice. The effects of sodium chloride in the glutamine reactions (Table III) are also inconsistent with a concentration effect. When the rate at 0° remained approximately constant (pH 6), the rate at −18° more than doubled. When the rate at 0° fell by 57% (pH 7), the rate at −18° remained constant.

Diketopiperazine reacts more rapidly with hydroxylamine at −18° than at −11° (Table IV), but hydrolysis of the penicillin β-lactam is more rapid at −8° than at −18°.[1] This suggests that the optimal temperature in the frozen systems may depend upon factors such as the interaction between the particular reactants involved and the crystalline structure of ice, which undergoes many transitions between 0 and −30°.[11]

Until recently little attention was paid to the chemical implications of the restricted mobility in many biological systems. Goldstein, *et al.*,[12] in studying the behavior of a water-insoluble trypsin derivative, stressed the effects of the "microenvironment," *i.e.*, such factors as "local" chemical composition, dielectric constant, and affinity for substrate. Factors of

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this kind may help explain the nature of reactions in frozen systems, which may, in turn, provide clues for explaining complex reactions in restricted biological systems. Moreover, since several bimolecular organic transformations in ice appear to be new examples of solid-state reactions, their characterization will require understanding of the reactant and product diffusion processes and the role of crystal imperfections, *e.g.*, lattice faults, vacant sites, and channels.

Several possible explanations have been offered for the enhanced reaction rates in ice: increased proton mobility,[1] imposition of a favorable positional orientation between reactants,[1] and participation of the ice crystal surface as replacement for a catalyst molecule.[5] Another important factor may be dielectric behavior, the dielectric constant of ice being markedly lower than that of water. This could promote aggregation of the catalyst through hydrogen bonding, resulting in a concerted attack such as found with covalently linked imidazole residues.[13]

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Isotuboflavine and Norisotuboflavine. Two New Alkaloids Isolated from *Pleiocarpa mutica* Benth.[1]

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Contribution from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts. *Received April 29, 1965*

Three new β-carboline derivatives were isolated from the stem bark of Pleiocarpa mutica Benth. One of them was identified as 1-carbomethoxy-β-carboline. For the other two, named isotuboflavine and norisotuboflavine, structures VIII and VII, respectively, were deduced, mainly on the basis of mass spectrometric evidence.

Since the reports[2-4] of the occurrence of hypotensically active alkaloids in the roots of *Pleiocarpa tubicina* Stapf and in the leaves, roots, and seeds of *Pleiocarpa mutica* Benth. these plants have been investigated in various laboratories[5-15] particularly by Schmid, *et al.*[5-12] During the past few years, more than 20 indole alkaloids have been isolated from these sources and the structures of most of them were determined.[6-13] The majority of the alkaloids possess the aspidofractine skeleton and the most prominent representative of this group is pleiocarpine (I).[6] In addition to these hexacyclic alkaloids there occur others, some of which are also found in other *Apocynaceae*, such as 1,2-dehydroaspidospermidine,[10] quebrachamine,[10] eburnamine,[5] and related compounds as well as alkaloids[12] with the akuammicine–condylocarpine carbon skeleton. Most recently[8] pleiocarpamine had been shown to represent a variant of the general pattern of indole alkaloids.

In addition to these indole and dihydroindole derivatives, two β-carboline alkaloids had been isolated: *P. mutica* gave flavocarpine (II),[13] and from the root bark of *P. tubicina* a very small amount of tuboflavine (III)[11] was obtained.

In the course of a detailed investigation of the alkaloids present in an extract of the stem bark of *P. mutica* we have isolated a few milligrams of three additional β-carboline alkaloids. The determination of their structures will be discussed in this paper.

On repeated careful chromatography of the crude extracts on alumina and silicic acid, four optically inactive ultraviolet fluorescing substances were isolated. One of these was identified as tuboflavine (III)[11] on the basis of spectral data and its melting point. Another component which also exhibited the typical ultraviolet spectrum of a β-carboline had a molecular weight of 226 and the infrared, n.m.r. and mass spectrum was most consistent with the presence of a carbomethoxy group. The compound was subsequently identified as 1-carbomethoxy-β-carboline (IV).[16] To our knowledge this compound has not yet been isolated from a natural source.

The two other substances had properties very similar to those of tuboflavine but did not seem to be identical with any alkaloid previously isolated from this plant material or any other source. Both were obtained in the form of yellow needles which melted at 282–284 and 262–264°, respectively. From a determination of the accurate mass of these compounds their elemental composition was deduced as $C_{15}H_{10}N_2O$ and $C_{16}H_{12}N_2O$,

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