the system during hydrolysis. This work will be reported in a subsequent paper.

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The Hydrolysis of Piperazine-2,5-dione*

Brian D. Sykes, E. Brock Robertson, H. B. Dunford, and Dennis Konasewich

ABSTRACT: The hydrolysis of piperazine-2,5-dione or diketopiperazine to glycylglycine was studied in 0.1 M HCl and 0.1 M NaOH over a range of temperatures. The values of ΔH^* and ΔS^* were found to be 20.6 kcal/mole and -14.7 cal deg^{-1} mole^{-1}, respectively, in acid, and 8.7 kcal/mole and -38.5 cal deg^{-1} mole^{-1}, respectively, in base. No ^{18}O exchange could be detected in proton-catalyzed hydrolyses at 111°. Arguments are presented which indicate that the rate-controlling step in the proton-catalyzed hydrolysis may be ring opening which is concerted with water addition. In the hydroxyl ion catalyzed hydrolysis, the rate-controlling step appears to be OH^- addition to the carbonyl group.

The results of some nuclear magnetic resonance (nmr) experiments are consistent either with a planar structure or rapidly flipping boat structure for diketopiperazine in aqueous solution. Whether acetylglycine or diketopiperazine is a better model for an infinitely long polyglycine chain is discussed.

Piperazine-2,5-dione, commonly called diketopiperazine (DKP),[1] has a ring structure which contains two peptide linkages (Figure 1). It has peculiar solubility properties, that in although it contains highly polar groups, it does not have a high solubility in polar solvents. It exhibits the expected low solubility in solvents of low polarity. The formol titration (Edward and Meacock, 1957) was used to follow the rate of

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[1] Abbreviations used in this work: DKP, diketopiperazine or piperazine-2,5-dione; GG, glycylglycine; nmr, nuclear magnetic resonance.

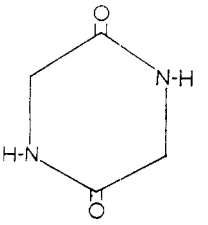

FIGURE 1: Structural formula for piperazine-2,5-dione or diketopiperazine.

hydrolysis of DKP over a wide range of acid concentrations at 61°. We report here on a study of the kinetics of its proton and hydroxyl ion catalyzed hydrolysis as a function of temperature, and the use of water 5% enriched in ^{18}O to determine whether any carbonyl oxygen exchange occurs during the course of the acid-catalyzed reaction. Since there is some question as to whether the DKP molecule is planar in solution, a few nuclear magnetic resonance (nmr) experiments were performed.

Experimental Section

The quality and treatment of all reagents are essentially as described elsewhere (Robertson *et al.*, 1964).

Procedure for Typical Experiments in Acid Solutions. A weighed amount of stock DKP solution of known density was added to a 100-ml volumetric flask and diluted to the mark with stock HCl solution in a bath thermostated at 25 ± 0.02°. Concentrations of DKP and HCl after mixing were calculated on the assumption that partial molal volume effects are negligible. Spectrophotometric analyses, discussed below and described in detail elsewhere (Robertson *et al.*, 1964), were also performed at 25°. Corrections of concentrations, to the temperature at which hydrolysis experiments were conducted, were made on the assumption that the solutions had the same coefficient of expansion as pure water. Typical concentrations in the reaction mixture were 0.002 M DKP and 0.1 M HCl.

Eleven 5-ml aliquots of the reaction mixture at 25° were pipetted into 14-mm Pyrex break-seal tubes. The sealed tubes were placed in a wire basket and lowered into a thermostated oil bath. Temperatures of the baths were 70.00 ± 0.02, 80.27 ± 0.02, 90.00 ± 0.02, 100.80 ± 0.03, and 111.20 ± 0.05°. The 101 and 111° baths had tightly fitting covers with small doors through which samples were admitted. Individual samples were removed at evenly, and accurately timed, intervals, quenched in ice water, labeled with a diamond pencil, and then placed in a freezer. Typically, the reaction was allowed to proceed until the absorptivity of the last sample was approximately twice the blank correction.

DKP and glycylglycine (GG) stock solutions for Beer's law controls were prepared by the above outlined procedure. These samples, of course, were not immersed in the heated oil baths and so were not placed in break-seal tubes.

Batch analyses, involving 36 samples at a time, were performed. These consisted of (1) two groups of eleven samples each from hydrolysis experiments, which were thawed and shaken thoroughly prior to analysis; (2) five DKP Beer's law controls; (3) five GG Beer's law controls; and (4) four blank samples, containing no DKP or GG but otherwise identical with the other samples in composition. All 36 samples were subjected to as identical treatment as possible. Two-milliliter aliquots of each sample were taken after thorough rinsing of the pipet. After the addition of all analytical reagents, these 2-ml samples were diluted to 10 ml.

Analytical Procedure. The analytical procedure is an adaptation of the ferric hydroxamate method. In order to minimize the correction for blanks and the interference from GG, the concentration of stock ferric chloride solution, used to develop the colored complex, was 0.185 M, the lowest concentration of ferric ion which had been tested. It was for this concentration of ferric ion that molar absorptivities of DKP and GG were reported previously, under otherwise optimum conditions for DKP analysis (Robertson *et al.*, 1964).

Two cells with quartz end windows, 65 mm long × 30 mm high × 5 mm wide, inside dimensions, were used for analyses of the acid-catalyzed reaction, performed on a Beckman DU spectrophotometer. Distilled water was used as the reference solution. A timer was started upon addition of ferric ion to the solution, which was shaken and allowed to stand 30 sec prior to commencement of the first absorptivity measurement. All absorptivity measurements were made at 540 mμ. A total of three absorptivity measurements were made on each sample, the time of each reading being recorded. Plots of absorptivity *vs.* time were made and the absorptivity indicated 2 min after ferric ion addition minus the mean blank was used for calculations in all cases.

Procedure for Experiments in Basic Solutions. The reaction was studied in 0.1 M NaOH. Because of the rapid rate of the base-catalyzed reaction compared to the acid-catalyzed reaction, measurements were restricted to 0 and 25°. Spectrophotometric measurements were made using 1-cm cells, rather than the 6.5-cm cells used in the acid-catalyzed studies. For the above reasons, several revisions were made in procedure. Stock solutions of DKP and NaOH were thermostated at the temperature at which the reaction was to be studied, either in an ice water bath or at 25 ± 0.02°. For the 0° experiments, appropriate density corrections were applied. A 15-ml portion of the DKP stock solution was placed in a 100-ml volumetric flask, which was filled to the mark with NaOH solution as rapidly as possible. The solution was shaken immediately, the time of commencement of shaking being taken as zero reaction time. The reaction mixture was kept in a thermostated bath. Quenching of the reaction was performed by the addition of a 2-ml aliquot of the reaction mixture to 2 ml of ice-cold 4 M hydroxylammonium chloride. A 2-ml portion of 4.05 M NaOH solution was added immediately, followed by immediate heating on the steam

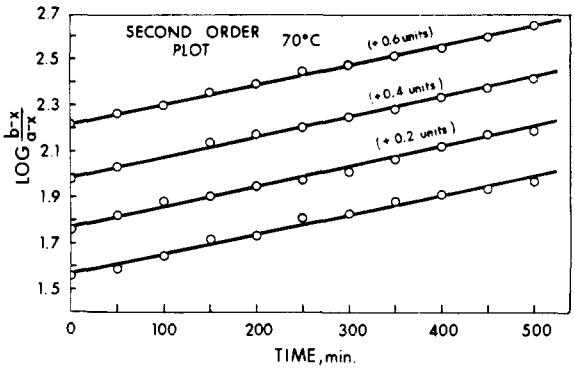

FIGURE 2: Second-order plots for the hydrolysis of diketopiperazine in 0.1 M HCl at 70°. The plots are separated by the addition of different logarithmic increments.

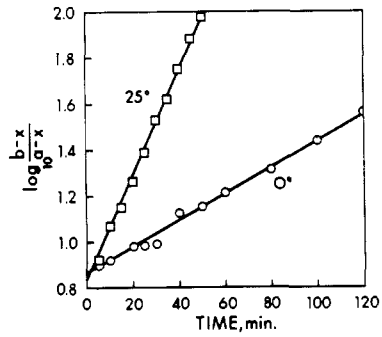

FIGURE 3: Second-order plots for the hydrolysis of diketopiperazine in 0.1 M NaOH at 0 and 25°.

bath to develop the hydroxamic acid. Thus, individual rather than batch analyses were performed.

Calculations. If $(a - x)$ and x are the total concentrations of DKP and GG, respectively, at time t (see Discussion), if A is the total absorptivity of the solution, and if α and β are the absorptivities of DKP and GG, respectively, then

$$A = \alpha(a - x) + \beta x \qquad (1)$$

Therefore, a plot of log $(b - x)/(a - x)$ *vs.* t, where $(b - x)$ is the concentration of catalyzing species, either H^+ or OH^-, at time t, is identical with a plot of log $(\beta b - \alpha b + \alpha a - A)/(\beta a - A)$ *vs.* t. Second-order rate constants, k, are obtained from the slopes of these plots from the relation

$$k = 2.303 \times \text{slope}/(b - a) \qquad (2)$$

Oxygen-18 Experiments. The water, 5% enriched in ^{18}O, was obtained from Fluka AG Chemische Fabrik, Switzerland. In a typical experiment, 0.02 g of pure DKP was added to 1 ml of enriched water, then 0.01 ml of 12 M HCl solution was added. The sample was then sealed in a 7-mm Pyrex tube and partially hydrolyzed at 111.2°. Experiments were conducted which resulted in 10, 20, 30, and 40% hydrolysis. After partial hydrolysis, the sample was emptied into a 10-ml beaker to which 3 ml of absolute ethanol was added. The sample was allowed to sit in a refrigerator overnight. The precipitated DKP was then filtered, washed twice with cold absolute ethanol, air dried, and then placed in a vacuum desiccator over P_2O_5 for 0.5 day. This procedure was found in control experiments to give 75–95% recovery of unhydrolyzed DKP, and to cause no precipitation of GG.

About 0.004 g of the recovered DKP was added to a break-seal Pyrex tube, evacuated, and sealed off. It was then placed on a carbon boat inside a quartz tube, which in turn was placed in a furnace at room temperature and evacuated. The furnace was turned on and when its temperature had reached 600°, as indicated by a chromel–alumel thermocouple located on the carbon boat, it was switched off. The heating period was about 20 min. The pyrolyzed sample was allowed to cool to room temperature in the furnace. A sample of DKP, 40% hydrolyzed in unenriched water, recovered, and pyrolyzed, was used as a control. The pyrolyzed samples were analyzed in a Metropolitan–Vickers MS-2 mass spectrometer, using 70-v electrons. Samples were scanned from *m/e* 12 to 70, and then the 44–46 and 28–30 peaks were scanned five times each for each sample.

Nuclear Magnetic Resonance Experiments. A spectrum of DKP in water was obtained on a Varian A-60 instrument. DKP deuterated on nitrogen was obtained by exchange with D_2O. Three spectra of deuterated samples in D_2O to which increasing amounts of H_2SO_4 were added were obtained.

Results

The mean of four determinations at each temperature of second-order rate constants, obtained from the proton-catalyzed experiments, are summarized in Table I. Each determination is the result of 11 sample analyses. Second-order plots of the 70° data are shown in Figure 2. Rate constants for the hydroxyl ion catalyzed studies are summarized in Table II; some of the

TABLE I: Second-Order Rate Constants, k, for the Hydrolysis of Diketopiperazine in 0.1 M HCl. Also Shown Are Standard Deviations in k, Computed from Four Independent Determinations of k at Each Temperature.

Temp (°C)	k (l. mole^{-1} min^{-1})
70.00 ± 0.02	(1.93 ± 0.01) × 10^{-2}
80.27 ± 0.02	(5.39 ± 0.14) × 10^{-2}
90.00 ± 0.02	(9.44 ± 0.17) × 10^{-2}
100.80 ± 0.03	(2.90 ± 0.05) × 10^{-1}
111.20 ± 0.05	(5.49 ± 0.27) × 10^{-1}

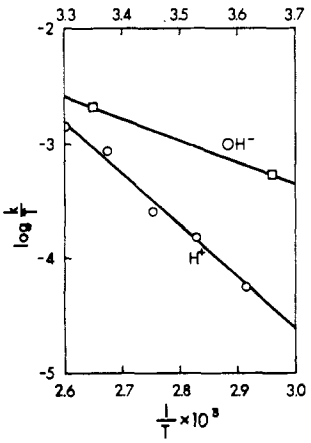

FIGURE 4: Plots of log k/T *vs.* $1/T$ for the hydrolysis of diketopiperazime. Upper $1/T$ scale refers to OH^- catalysis; lower scale to H^+ catalysis.

TABLE II: Second-Order Rate Constants, k, Expressed in l. $mole^{-1}$ min^{-1}, for the Hydrolysis of Diketopiperazine in 0.1 M NaOH.

0.0 ± 0.2°	25.0 ± 0.02°
0.149	0.634
0.151	0.636

second-order plots are shown in Figure 3. The four values of k reported in Table II are based on 5, 11, 10, and 10 sample analyses. Plots of log k/T *vs.* $1/T$, where k is expressed in l. $mole^{-1}$ min^{-1}, for the proton and hydroxyl ion catalyzed results, are shown in Figure 4. From the slopes and intercepts of these plots, with an appropriate correction for the units of time, the ΔH^* and ΔS^* values shown in Table III were obtained by the method of least squares.

All mass spectra showed the predominant peaks to be at mass to charge ratios (m/e) of 28/1 and 44/1, corresponding to the peaks of $C^{16}O$ and $C^{16}O_2$, respectively. For all samples analyzed, the ratio of the peak heights at m/e 30 and 28, corresponding to $C^{18}O/C^{16}O$, was 6.4×10^{-3} ($\pm 0.4 \times 10^{-3}$ standard deviation). If the peak at m/e 28 is caused by $C^{16}O$ only and if k(exchange)/k(hydrolysis) = 1, then the 30/28 peak height ratio would be expected to increase to about 1.6×10^{-2} for samples hydrolyzed in $H_2{}^{18}O$. Similarly, for all samples analyzed, the ratio of peak heights 44/42, corresponding to $C^{16}O^{18}O/C^{16}O_2$, was 4.8×10^{-3} ($\pm 0.4 \times 10^{-3}$ standard deviation).

From the nmr spectrum obtained in water, a small singlet at τ 6.02 was tentatively identified as methylene hydrogen. The remaining peaks are presumably hydrogen exchanging between nitrogen and solvent water and the spinning side bands of water. However, this interpretation was not entirely clear, mainly because the side bands of water interfered with the methylene peak. The three deuterated samples gave sharp singlets at τ = 5.97, 5.97, and 6.00, which were assigned to methylene hydrogen, as before, and a peak which increased in size and shifted to lower field on addition of acid, and hence must be water.

TABLE III: Kinetic Parameters for the Proton and Hydroxyl Ion Catalyzed Hydrolysis of Diketopiperazine and Related Compounds.

	$-\Delta S_1^*$ (cal deg^{-1} $mole^{-1}$)	ΔH^* (kcal/mole)
A. Proton catalysis		
Diketopiperazine	14.7	20.6
Acetylglycine[a]	16.6	21.2
Diglycylglycine[b]		
gg-g[c]	19.2	20.4
g-gg[c]	21.3	20.6
Other tripeptides[b]		
AB-C[c]	21.8 ± 1.4	20.1 ± 0.4[d]
A-BC[c]	23.6 ± 1.3	20.3 ± 0.5[d]
Glycylglycine[a]	24.0	20.3
Other dipeptides[a]	25 ± 2	20.6 ± 1[d]
B. Hydroxyl ion catalysis		
Diketopiperazine	38.5	8.7
Tripeptides[e]	40 ± 3	14 ± 2[d]
Glycylglycine[a]	26.8	16.9

[a] Lawrence and Moore (1951). [b] Long and Truscott (1963, 1965). [c] C-terminal on the right. [d] Mean deviation for several different compounds. [e] Hartmann *et al.* (1962).

Discussion

Since the pK_a of the peptide linkage is probably below −4 (Edward and Meacock, 1957), there would be negligible protonation of DKP in 0.1 M HCl. The validity of the second-order rate law to near completion of the hydrolysis reaction confirms that the reaction scheme

$$DKP + H^+ + H_2O \rightarrow GGH^+$$

is adequate. Subsequent hydrolysis of GG is too slow to be of importance. The data of Table III indicate that DKP is hydrolyzed 100 times faster than GG in acid (Lawrence and Moore, 1951) and, within experimental error, this factor is temperature independent. As is also shown in Table III, the enthalpy of activation for proton-catalyzed hydrolyses is remarkably constant for dipeptides (Lawrence and Moore, 1951), tripeptides (Long and Truscott, 1963, 1965), and DKP. Since the entropy of activation averages two units more negative for N-terminal compared to C-terminal peptide bond cleavage in tripeptides, the charge on the amino group

has an inhibiting effect. By comparison of the reactivities of di-GG and GG with acetylglycine, a ΔS^* value of about -16.6 cal deg^{-1} mole^{-1} for the C-terminal peptide cleavage in an infinitely long polyglycylglycine is predicted (Long and Truscott, 1965). However, the peptide linkage is more basic in acetylglycine than in the peptides and DKP (Edward and Meacock, 1957). In DKP, despite the absence of charge, the peptide linkages are more acid than in acetylglycine because of the acidifying effect of the peptide groups on each other. The enhancement in acidity, or some related factor, may be greater in DKP than in a linear peptide chain because of the planar or near-planar structure of DKP in solution. Although DKP is known to be planar in the crystalline state (Corey, 1938; Vainshtein, 1955), its conformation in solution is not clearly established. Our nmr results are consistent either with a planar structure or a rapidly flipping boat structure (Edward, 1955). Another factor to be taken into account is the fact that peptide linkages in DKP have a *cis* configuration, whereas the preferred configuration in a polypeptide chain in *trans*. Until the effects of these factors can be evaluated precisely, it is not possible to state whether DKP or acetylglycine is a better model of an infinitely long polyglycine chain. A comparative study under conditions where the peptide linkage is completely protonated would be of interest (Lane, 1964).

The fact that all species listed in Table III, including DKP, have the same energy barrier to proton-catalyzed hydrolysis suggests that all protonated peptide linkages, regardless of parent compound, have equal energy, and all have comparable transition states. The apparent absence of ^{18}O exchange in acid indicates that k(hydrolysis) is appreciably greater than k(exchange), and the less negative entropy of activation for DKP hydrolysis is consistent with bond rupture, and hence ring opening, being part of the rate-controlling step. The latter two statements would indicate that addition of water to the protonated peptide linkage is concerted with the bond rupture process. The absence of ^{18}O exchange is contrary to a prediction based on the w and w* criteria (Bunnett, 1961), and emphasizes that any mechanistic criteria based on the Hammett acidity function is applicable only to compounds which have activity coefficient behavior comparable to that of Hammett indicators.

The OH^- catalyzed hydrolysis of DKP has roughly the same entropy of activation as a tripeptide, but a much lower activation enthalpy (Hartmann *et al.*, 1962) (Table III). Since the modifying influence of the proton on the peptide linkage is now absent, factors such as strain in the near-planar DKP structure, and the higher energy of the *cis* configuration, now lower the energy barrier to the hydrolysis of DKP. The same entropy of activation indicates that bond rupture is not part of the rate-controlling step, which consists solely of OH^- addition to form a tetrahedral intermediate or transition state. The much more rapid rate of hydrolysis of DKP compared to that of peptide linkages in linear molecules confirms the arguments (Bender, 1960) that attack at the carbonyl carbon atom does not occur from the rear of the —C═O group, which is blocked in DKP, but occurs at right angles to this group.

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