activity was dependent on the surface pressure of a film of acetylcholinesterase, and the maximum activity observed was at a surface pressure of 10 dynes/cm (2). This activity was equal to 29% of the activity of the same amount of enzyme in solution. The fraction of the retained enzyme activity on adsorption at an air–water interface in the case of trypsin varied from 0.125 to 0.35, depending on the surface concentration of the enzyme (3).

This study demonstrated the effectiveness of the subphase exchange technique for investigating the catalytic activity of enzymes spread at an air–water interface, without interference of bulk reaction. Future reports will discuss the effects of various lipids on the catalytic activity of surface-spread malate dehydrogenase.

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Kinetics and Mechanisms of Hydrolysis of 1,4-Benzodiazepines III: Nitrazepam

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Abstract □ The hydrolysis of nitrazepam involves a two-step sequential mechanism. The intermediate is the ring-opened compound resulting from scission of the azomethine bond. The final products are glycine and 2-amino-5-nitrobenzophenone. Recyclization of the intermediate to nitrazepam occurs at pH values above the pKa of the intermediate, in the pH region where the amino group of the intermediate is not protonated. As opposed to chlordiazepoxide and oxazepam, the initial hydrolysis step occurs at the 4,5-bond, not at the 1,2-amide linkage. This difference is attributed to a preferential activation for hydrolysis of the azomethine linkage by the nitro group. The hydrolysis involves an uncatalyzed reaction, specific acid–base catalysis, and general acid–base catalysis for acetate and phosphate buffers.

Keyphrases □ 1,4-Benzodiazepines—nitrazepam, kinetics and mechanisms of hydrolysis □ Nitrazepam—kinetics and mechanisms of hydrolysis □ Hydrolysis—nitrazepam, kinetics and mechanisms □ Anticonvulsants—nitrazepam, kinetics and mechanisms of hydrolysis

Nitrazepam, 1,3-dihydro-7-nitro-5-phenyl-2*H*-1,4-benzodiazepin-2-one, belongs to the 1,4-benzodiazepine class of tranquilizing agents. This compound is centrally active (1), surpassing diazepam, a popular benzodiazepine, in anticonvulsant activity (2).

Preceding publications (3, 4) in this series reported the hydrolysis kinetics of 7-chloro-1,4-benzodiazepines. The present study concerned the hydrolysis of the 7-nitro analog, nitrazepam.

EXPERIMENTAL

Materials—Compound purity was verified by TLC. Nitrazepam[1] and 2-amino-5-nitrobenzophenone[1] were used as received.

Buffer Solutions—All buffer solutions were made with deionized, distilled water. They were adjusted to an ionic strength of 1.0 with sodium chloride, except in ionic strength effect studies where they were adjusted to ionic strengths other than 1. The pH at the temperature of the runs was measured with a digital pH meter[2] equipped with high temperature electrodes.

The buffer systems used were: pH 1.0–3.0, hydrochloric acid; pH 3.2–5.6, acetate; pH 6.4–7.4, phosphate; pH 7.9–9.5, borate; and pH 10.1–11, sodium hydroxide.

Kinetic Measurements—The kinetic studies of the hydrolysis of nitrazepam were followed spectrophotometrically. Details of the specific procedures were given previously (3) and are summarized as follows.

A stock solution of nitrazepam (6.3×10^{-3} *M*) in ethanol was diluted in an appropriate buffer to a final concentration for the kinetic run of 2.4×10^{-5} *M*. The reaction flasks (light protected) were maintained in a constant-temperature oil bath[3] controlled within 0.1° at selected temperatures between 70 and 85°. Samples were withdrawn at suitable time intervals and cooled to room temperature by quenching in an ice water bath. Visible and UV spectrophotometric measurements were made with a double-beam spectrophotometer[4].

Isolation and Identification—Thin-layer chromatograms were made on 20 × 20-cm glass plates coated with a 250-μm layer of silica gel GF_{254}. After lyophilization, the samples were dissolved in chloroform, spotted, and developed in a closed tank for approximately 1 hr with dioxane–benzene–hexane–7.4 *M* NH_4OH (45:50:70:5). The solvent front was allowed to travel 17 cm from the origin. After drying, the plates were visualized using a short wavelength UV lamp and then sprayed with ninhydrin aerosol (0.5%) to produce visible spots. Nitrazepam, 2-amino-5-nitrobenzophenone, and glycine were spotted as reference standards. An intermediate was isolated by preparative TLC and characterized by its IR[5], NMR[6], and high-resolution mass[7] spectra.

[1] Hoffmann–La Roche, Nutley, N.J.

[2] Model 701, Orion Research, Cambridge, Mass.

[3] Sargent model SW equipped with Sargent thermonitor model ST, R. L. Sargent Co., Dallas, Tex.

[4] Model 124, Coleman Instruments Division, Perkin-Elmer Corp., Maywood, Ill.

[5] Model IR8, Beckman Instruments, Fullerton, Calif.

[6] Model C60HL, Jeol, Medford, Mass.

[7] CEC-21-110, DuPont Instruments, Wilmington, Del.

Table I—Apparent First-Order Rate Constant, 10^4 k (in Minutes^{-1}), for Nitrazepam at 75°, μ = 1.0

pH	Buffer		k_1	k_2	r_1	r_2
		[HCl]				
0.93		0.1000	599 ± 13.5	9.04 ± 1.33	0.99	0.98
1.70		0.0158	499 ± 5.4	3.06 ± 0.03	0.99	0.99
2.60		0.0025	365 ± 6.4	1.70 ± 0.01	0.99	0.99
3.03		0.0010	363 ± 17.4	1.51 ± 0.07	0.99	0.99
	[CH_3COOH]	[CH_3COO^-]				
3.24	0.0957	0.0043	—	1.60 ± 0.05	—	0.99
4.00	0.0761	0.0239	—	0.88 ± 0.03	—	0.99
4.85	0.0322	0.0678	—	0.67 ± 0.01	—	0.99
5.64	0.0081	0.919	—	0.85 ± 0.10	—	0.96
	[$H_2PO_4^-$]	[HPO_4^{2-}]				
6.47	0.0275	0.0391	—	0.209 ± 0.09	—	0.99
7.40	0.0049	0.0617	—	19.47 ± 0.19	—	0.99
	[H_3BO_3]	[$H_2BO_3^-$]				
7.93	0.103	0.029	—	43 ± 0.18	—	0.99
8.52	0.061	0.110	—	196 ± 4.04	—	0.99
9.27	0.008	0.100	—	428 ± 12.16	—	0.99
9.53	0.003	0.100	—	458 ± 23.74	—	0.99
		[NaOH]				
10.18		0.01	—	1957 ± 210	—	0.98

RESULTS AND DISCUSSION

Spectral Changes and Rate Constant Determinations—The kinetics of hydrolysis of nitrazepam were monitored by following the change in absorbance, A, of the visible and/or UV chromophore as a function of time. The pKa values for nitrazepam are 3.2 and 10.8 (5). No pKa was found for 2-amino-5-nitrobenzophenone in the pH region of the present study. For pH values below the pKa of 3.2, two apparent reaction steps were observed. The first was characterized by an absorbance decrease at λ_{max} 278 nm with isosbestic points at 252 and 315 nm. The second showed an absorbance decrease at λ_{max} 265 nm with the formation of a new absorbance band with λ_{max} 364 nm.

For pH values intermediate to pKa_1 and pKa_2, only one reaction step was observed spectrally. The spectral changes were similar to those observed for the second hydrolysis step for pH values below pKa_1. For pH values above pKa_2, the single reaction step observed was characterized by a red shift from λ_{max} 364 to 380 nm. The spectrum at the time infinity point was identical to the spectrum of 2-amino-5-nitrobenzophenone throughout the entire pH region.

The logarithms of the difference in the final absorbance, A_∞, and the absorbance at any time, A_t, at the desired wavelength were plotted against time. The apparent first-order rate constants were calculated from the slopes of the linear segment according to:

$$\ln(A_t - A_\infty) = \ln(A_0 - A_\infty) - kt \qquad \text{(Eq. 1)}$$

where A_0 is the absorbance at zero time, and k is the apparent first-order rate constant. Absorbance changes in the visible region (370 nm) were used to calculate the apparent first-order rate constants for all pH values above the first pKa. For pH values below pKa_1, plots made according to Eq. 1 were biphasic, indicative of a two-step hydrolytic reaction in this pH region. For these data, the "feathering" technique was utilized to determine the sequential apparent first-order rate constants for the first and second reaction steps. Typical first-order plots for the hydrolysis of nitrazepam are shown in Fig. 1. The rate constants derived from these plots are presented in Table I.

Based on the observed spectral and kinetic behavior of nitrazepam (I), a two-step sequential hydrolysis pathway could be proposed (Scheme I). A reversible first-step hydrolytic ring opening of the benzodiazepine nucleus is followed by a second hydrolytic step, leading to the formation of glycine and a substituted benzophenone derivative.

Log k–pH Profiles—The log k–pH profile for nitrazepam was constructed at 75° with the data in Table I. For pH values below pKa_1, the first reaction step can be described by:

$$k_{obs} = k_{H_2O} f_{NH} \qquad \text{(Eq. 2)}$$

or by its kinetic equivalent:

$$k_{obs} = k_H[H^+]f_N \qquad \text{(Eq. 3)}$$

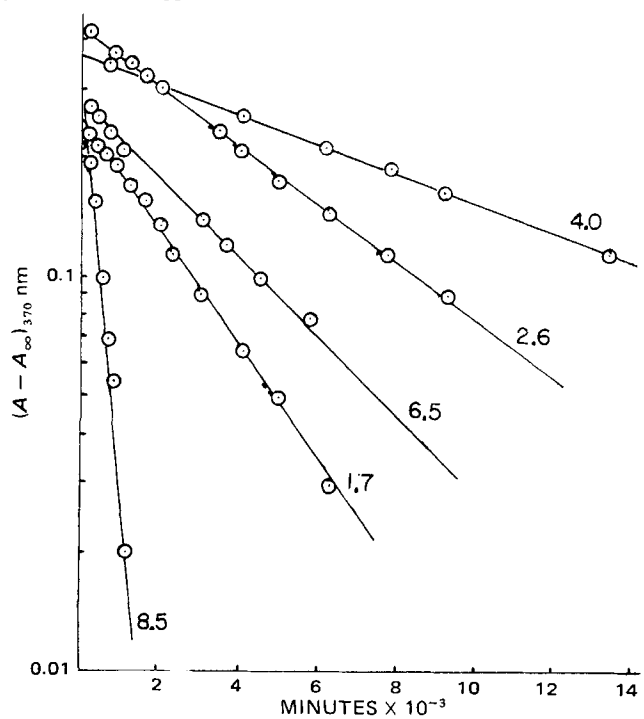
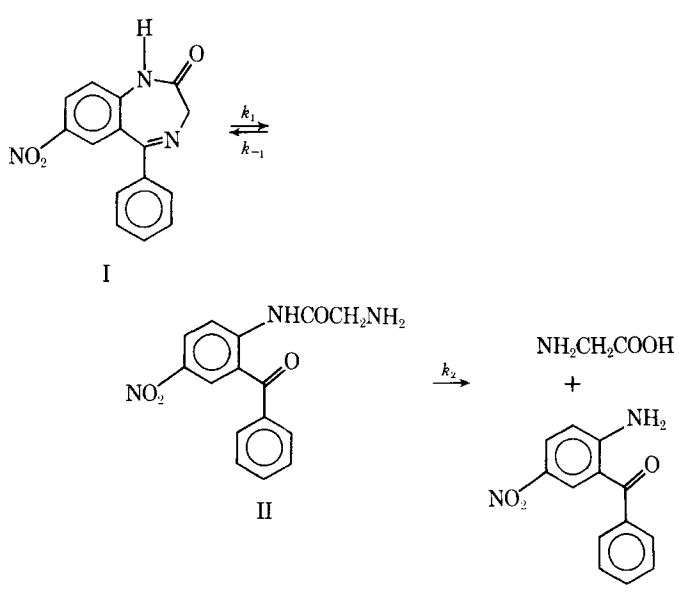


Figure 1—*Typical apparent first-order plots for the hydrolysis of 10^{-5} M nitrazepam as a function of pH.*



Scheme I

Table II—Apparent First-Order Rate Constants, 10^4 k (in Minutes^{-1}) for Hydrolysis of Nitrazepam at Various Buffer Concentrations

pH	Total Buffer Concentration in 1X	1X	2X	3X	4X	k_0
4.74	[CH_3COOH][CH_3COO^-] 0.100	0.63	0.74	1.0	1.17	0.41
6.37	[$H_2PO_4^-$][HPO_4^{2-}] 0.066	2.52	3.08	3.42	3.56	2.2

The terms f_{NH} and f_N are the fractions existing as protonated and deprotonated nitrazepam, respectively.

The rate constants that provided the best fit are $k_{H_2O} = 0.054$ min^{-1} and $k_H = 54$ min^{-1} M^{-1}. The kinetic pKa at 75° was 3.0, in agreement with the reported pKa_1 of nitrazepam at room temperature.

The log k–pH profile for the second reaction step exhibited five distinct components. At low pH values, the kinetics indicated hydrogen-ion catalysis on the protonated intermediate H_2S, followed by water attack on the neutral intermediate, HS. Uncatalyzed hydrolysis on the neutral intermediate, or the kinetic equivalent of hydroxide-ion attack on the protonated species, was observed over the neutral pH region.

The hydrolysis kinetics of nitrazepam in the basic pH region indicated hydroxide-ion catalysis on the double deprotonated intermediate, S, followed by water attack on the deprotonated species or its kinetic equivalent of hydroxide-ion catalysis on the neutral species. The apparent first-order rate constant at any pH can be quantitatively described by:

$$k_{obs} = k_H[H^+]f_{H_2S} + k_{H'}[H^+]f_{HS} + k_{H_2O}f_{HS} + k_{OH}[OH]f_{HS} + k_{OH'}[OH^-]f_S \quad \text{(Eq. 4)}$$

or the following substituted kinetic equivalents:

$$k_{H'}[H^+]f_{HS} = k_{H_2O}'f_{H_2S} \quad \text{(Eq. 5)}$$

$$k_{H_2O}f_{HS} = k_{OH''}[OH^-]f_{H_2S} \quad \text{(Eq. 6)}$$

$$k_{OH'}[OH^-]f_{HS} = k_{H_2O}''f_S \quad \text{(Eq. 7)}$$

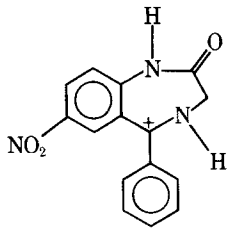
The bimolecular rate constants that provided the best fit are $k_H = 0.0088$ min^{-1} M^{-1}, $k_{H'} = 1.88$ min^{-1} M^{-1}, $k_{H_2O} = 0.00015$ min^{-1}, $k_{OH} = 1.52 \times 10^5$ min^{-1} M^{-1}, and $k_{OH'} = 247$ min^{-1} M^{-1}.

From the log k–pH profile, the two kinetic pKa's were calculated as 4.1 and 8.8 and were assigned to the amino nitrogen and the amide group of the intermediate, respectively. The experimental data points with the theoretical curves calculated from Eqs. 2 and 4 are shown in Fig. 2.

Isolation and Identification—Samples withdrawn at appropriate times were subjected to TLC analysis. At pH values below the pKa_1 of nitrazepam, TLC showed an increase in intensity of the yellow 2-amino-5-nitrobenzophenone spot (R_f 0.43) and a decrease in the relative intensity of the nitrazepam spot (R_f 0.22). A compound (R_f 0.17) appeared transiently and was attributed to a reaction intermediate. For pH values above the pKa_1 of nitrazepam, no intermediate was observed by TLC. The spectral changes in this pH region also demonstrated a single isosbestic point, indicative of a one-to-one transformation.

The intermediate was isolated as a yellow solid by preparative TLC. Mass spectrometric analysis of the intermediate demonstrated a possible molecular ion at 18 mass units above the molecular weight of nitrazepam. A compound with this molecular weight could result from hydrolysis of either the 1,2-amide bond or the 4,5-azomethine bond. The NMR spectrum did not show a characteristic carboxylic acid absorption. Since the acid group would result from heterolysis of the amide, the structure resulting from hydrolysis of the azomethine bond was tentatively assigned as II in Scheme I.

Corroborating evidence for this structural assignment was obtained by studying the reversibility of the reaction leading to II. Prior reports (6, 7) showed that, in 1,4-benzodiazepin-2-one hydrolysis, the recyclization of the protonated opened-ring intermediate resulting from scission of the 1,2-amide linkage is quite facile. Additionally, the deprotonated form does not undergo recyclization (3). The opposite was found for recyclization of intermediate II. When heated at 75° for 5 min, the intermediate was shown spectrally to revert to nitrazepam at pH values greater than 5. At pH values below the first pKa of the intermediate, no recyclization was indicated. Indeed, at the higher pH values, II was not observable by either TLC or spectral measurements. If the intermediate is of the benzophenone structure (II), then protonation of the primary amine at pH values below 4 would decrease its nucleophilicity. This, in turn, would decrease the rate of recyclization, in agreement with the experimental results showing rapid recyclization only at pH values above 5.



IV

Hydrolytic Mechanisms—The preceding kinetic and TLC data are mechanistically interpretable according to Scheme I. The first hydrolytic reaction step is only reversible at pH values above the pKa_1 of the intermediate. Initially, the 4,5-bond is hydrolyzed, leading to the ring-opened compound, intermediate II. Although models show that this bond is sterically hindered to nucleophilic attack relative to the amide bond, protonation of the azomethine nitrogen should electronically favor attack at the 5-position. This conclusion is based on possible resonance contribution shown as Structure IV.

However, the 7-chloro-1,4-benzodiazepines chlordiazepoxide (3) and oxazepam (4), also capable of 4-nitrogen protonation to lead to a positive charge resident on the 5-position by resonance, show initial 1,2-bond hydrolysis preferentially. The difference noted between these 7-substituted analogs can be interpreted using the Hammett relationship:

$$\log k/k_0 = \sigma\rho \quad \text{(Eq. 8)}$$

where k and k_0 are the rate constants for hydrolysis of the substituted and unsubstituted compounds, respectively; σ measures the electron-attracting ability of the substituents; and ρ is a parameter defined by the reaction type. The σ values for chloro and nitro substituents are σ_{Cl}^m 0.37 and $\sigma_{NO_2}^m$ 0.71 for *meta*-substitution and σ_{Cl}^p 0.23 and $\sigma_{NO_2}^p$ 0.78 for *para*-substitution (8). Upon substitution of these values into Eq. 8, the Hammett relationship can be written as:

$$\log (k_{Cl}^p/k_{Cl}^m) = -0.14\rho \quad \text{(Eq. 9)}$$

where k_{Cl}^p and k_{Cl}^m are the rate constants for the hydrolysis of *p*- and *m*-chloro-substituted positions, respectively. The equation indicates that the *m*-chloro-substituted position is less reactive than the *p*-chloro-substituted position, assuming a negative ρ value due to electronic requirements for acid-catalyzed hydrolysis. Thus, initial 1,2-bond hydrolysis would be favored. For the 7-nitro compound, the Hammett equation operable is:

$$\log (k_{NO_2}^p/k_{NO_2}^m) = 0.07\rho \quad \text{(Eq. 10)}$$

The *meta*-position (azomethine) hydrolysis would be slightly favored, again assuming a negative ρ.

Stability Parameters—To provide a complete profile of the stability of nitrazepam, hydrolysis kinetics as a function of buffer concentration, ionic strength, and temperature were investigated. Buffer catalysis was studied according to the relationship:

$$k_{obs} = k_0 + \left(\frac{k_{HA}[H^+]}{K_a} + k_A\right)[A^-] \quad \text{(Eq. 11)}$$

where K_a is the dissociation constant for the buffer species, k_0 is the hydrolysis rate constant in absence of buffer catalysis, and k_{HA} and k_A are the rate constants for buffer catalysis of the acid buffer species, HA, and its conjugate base, A^-, respectively. Buffer effects were investigated by changing the original buffer concentration fourfold in a gradient manner. Table II gives the results of these studies on the rate constants

Table III—Apparent First-Order Rate Constants, 10^4 k (in Minutes^{-1}) for Hydrolysis of Nitrazepam at Various Ionic Strengths.

pH	k_i	0.1	0.2	0.4	0.8	Q
0.98	k_1	973.5	1158.9	870.0	776.0	No relation
	k_2	6.06	7.3	8.05	9.04	0.37
4.8	k_2	0.52	0.52	0.61	0.58	No relation

Table IV—Apparent First-Order Rate Constants (10^4 k in Minutes^{-1}) and Thermodynamic Parameters for Hydrolysis of Nitrazepam at pH 0.93

	85°	80°	75°	70°	E_a	ln P	ΔH_a	ΔS_a	ΔF
k_1	1418.0	1166.5	599.5	550.3	17.0	22.0	16.3	−7.0	22.3
k_2	14.0	12.2	9.0	6.6	12.5	11.0	11.8	−38.9	25.5

for hydrolysis of nitrazepam. Both acetate and phosphate buffers catalyzed the hydrolysis.

The kinetics of hydrolysis were carried out for different ionic strengths from 0.1 to 1.0 at pH 0.98 and 4.80. The apparent first-order rate constants are given in Table III. No significant ionic strength effect was observed for the k_1 step at pH 0.98 and the k_2 step at pH 4.80. A positive ionic strength effect was observed for the k_2 step at pH 0.98. The use of the Bronsted–Bjerrum equation together with the extended Debye–Huckel equation yields:

$$\log k = \log k_0 + 2QZ_AZ_B \sqrt{\mu}/(1 + \sqrt{\mu}) \qquad \text{(Eq. 12)}$$

where k is the apparent first-order rate constant, k_0 is the rate constant at zero ionic strength, Q is a constant equal to 0.557 (9) at 80°, Z_A and Z_B are the charges of the reacting molecules, and μ is the ionic strength. The ionic strength effect plot for k_2 of pH 0.98 shows a Q value of 0.37. This result suggests that the hydrolysis at pH 0.98 involves a reaction between molecules of like univalent charge.

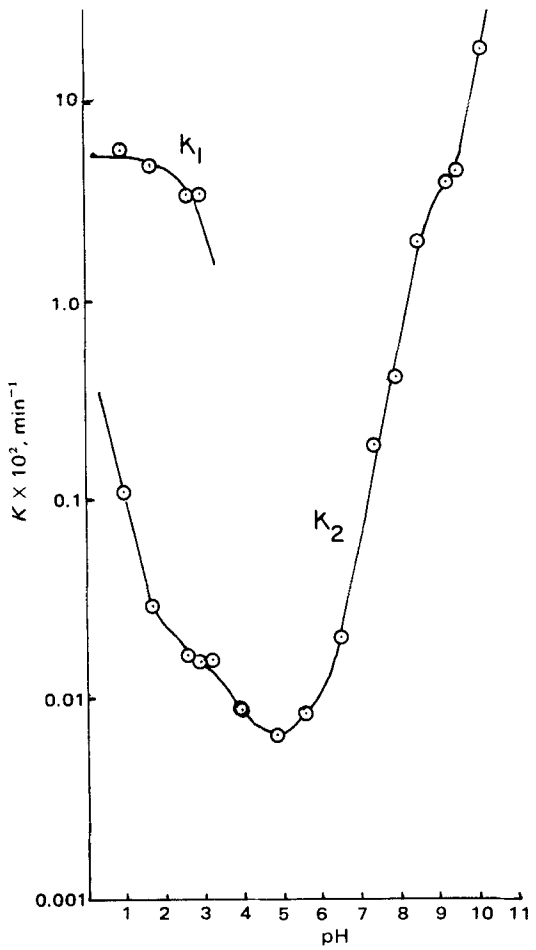


Figure 2—*Log* k–*pH profile for the hydrolysis of* 10^{-5} M *nitrazepam at 75°,* μ = *1.0. The* k_1 *and* k_2 *refer to the first and second reaction steps, respectively.*

The Arrhenius parameters for the hydrolysis of nitrazepam were obtained from the slope and intercept of a plot of the logarithm of the apparent first-order rate constant, k, *versus* the reciprocal of the absolute temperature, T, shown by:

$$\ln k = \ln P - E_a/RT \qquad \text{(Eq. 13)}$$

where R is the gas constant equal to 1.987 cal deg^{-1} mole^{-1}; ln P is related to the entropy of activation, ΔS_a, by:

$$\Delta S_a = R[\ln P - \ln (KT/h) - 1] \qquad \text{(Eq. 14)}$$

where h and K are the Planck and Boltzman constants, respectively; and E_a is related to the enthalpy of activation of the hydrolysis, ΔH_a, by:

$$\Delta H_a = E_a - RT \qquad \text{(Eq. 15)}$$

The Arrhenius parameters for the hydrolysis of nitrazepam at pH 0.93 are given in Table IV. These values will allow stability prediction in this pH region where degradation is rapid.

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