Acyl Transfer Reactions from and to the Ureido Functional Group. I. The Mechanisms of Hydrolysis of an *O*-Acylisourea (2-Amino-4,5-benzo-6-oxo-1,3-oxazine)

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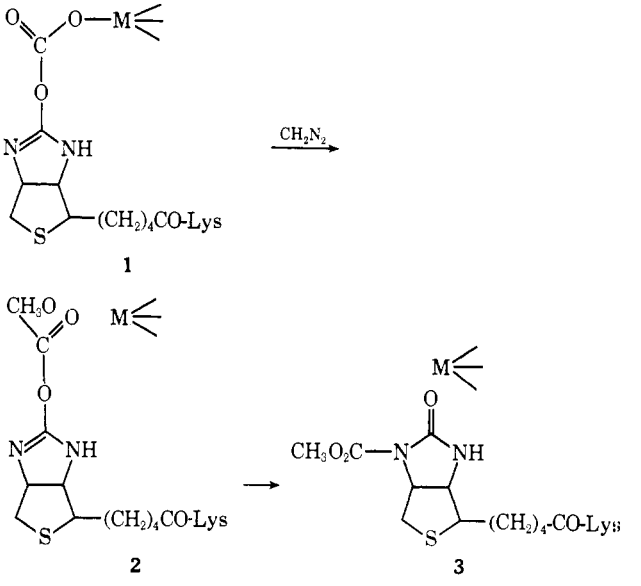
Abstract: The hydrolysis of 2-amino-4,5-benzo-6-oxo-1,3-oxazine (**6**) has been studied from pH 0.5 to 12.6 (30°, H_2O solvent, μ = 1.0). The pH–k_{obsd} profile for the hydrolysis of **6** to *o*-ureidobenzoic acid (**8**) can be separated into two distinct regions: (a) at pH values from 5 to 0.5, k_{obsd} is proportional to the mole fraction of protonated **6** [*i.e.*, $k_{obsd} = k_1'a_H/(K_{a_1} + a_H)$]; and (b) at pH values of 8–12 the k_{obsd} constants first rise and then fall with increase in pH to provide a sharp "bell-shaped" profile. To describe the complete pH–k_{obsd} profile, four species of **6** in acid–base equilibria are required [**6a**, **6b**, **6c**, and **6d**]. The presence of the four species was established by spectrophotometric titration, which also provided the associated apparent dissociation constants [**6a** → **6b** (pK_{a_1} = 3.10), **6b** → **6c** (pK_{a_2} = 10.61), **6c** → **6d** (pK_{a_3} = 9.21)]. The spectrophotometrically determined pK_a's were found to be identical with the kinetically determined pK_{app}'s required to fit the pH–k_{obsd} profile. The sharpness of the bell-shaped region of the pH–k_{obsd} profile is attributed to the unusual observation that $pK_{a_2} > pK_{a_3}$. The structures assigned to the species in solution are as follows: (a) **6a** is the conjugate acid of **6**; (b) **6b** is **6**; (c) **6c** arises from **6** by loss of a proton and ring opening to yield the monoanion of *o*-carboxyphenylcyanamide; and (d) **6c** then loses an additional proton to provide the dianionic species of *o*-carboxyphenylcyanamide (**6d**). Due to the fact that $pK_{a_2} > pK_{a_3}$, **6c** is never present in greater than 10% mol fraction of **6** at any pH. The disodium salt of **6d** was isolated and characterized by elemental analysis (for Na^+) and ir spectroscopy. The overall equilibrium of **6a** ⇄ **6d** is readily reversible. The open-ring species **6c** and **6d** are refractory to hydrolysis. The hydrolysis of **6a** is pH independent and characterized by a deuterium-solvent kinetic-isotope effect ($k_1^{H_2O}/k_1^{D_2O}$) of 2.0 and represents general base assisted attack by water of water at the acyl carbonyl carbon. Hydrolysis of **6b** is also associated with a small spontaneous rate constant. The ascending leg of the bell is associated with an increase in [HO^-] while [**6b**] is essentially constant and the descending leg due to the conversion of **6b** → **6d** through simultaneous loss of two protons and ring opening.

The nucleophilicity of the ureido group toward the acyl carbonyl carbon is of importance in the mechanism of biotin action (*i.e.*, attack of the imidazolidone moiety upon activated CO_2). Transfer of the acyl group from the ureido functional group is an important feature of both biotin action and the synthesis of peptide bonds *via* the carbodiimide reaction. This study and those described in the accompanying two manuscripts deal with acyl transfer reactions from and to the ureido functional group.

The formation of a CO_2 adduct at the oxygen of an enzyme bound isourea form of biotin cannot be excluded as the process leading to the intermediate enzyme–biotin–CO_2 in carboxylase reactions. Unquestionably, the diazomethane trapping experiments of Lynen, Knappe, and coworkers[3] established the trapped product to contain the carbomethoxy group at the 1′-nitrogen of biotin. That the trapped product might well have arisen *via* the isoimide rearrangement of Scheme I has apparently not been considered.[4] In the hypothetical sequence of Scheme I, species **1** would represent biotin-bound CO_2, **2** the initially trapped product, and **3** the isolated product formed by

Scheme I



way of a four-center N → O acyl transfer reaction. The rearrangement reaction of **2** → **3** would be anticipated on the basis that the configuration of the isoimide moiety of **2** is syn with the nonbonded electron pair of nitrogen in the proper steric alignment for attack upon the carbomethoxy group. Isoimides are rarely isolable unless stabilized by a special feature such as an anti configuration.[5] The four-center rear-

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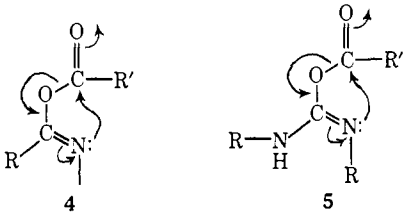
(2) To whom inquiries should be addressed.

(3) (a) J. Knappe, E. Ringelmann, and F. Lynen, *Biochem. Z.*, **335**, 168 (1961); (b) J. Knappe, H. G. Schlegel, and F. Lynen, *ibid.*, **335**, 101 (1961); (c) F. Lynen, J. Knappe, E. Lorch, G. Jutting, E. Ringelmann, and J. P. Lachance, *ibid.*, **335** 123 (1961); (d) J. Knappe, E. Biederbick, and W. Bruemmer, *Angew. Chem.*, **74**, 432 (1962); (e) J. Knappe, B. Wenger, and U. Wiegand, *Biochem. Z.*, **337**, 232 (1963).

(4) For further discussion see: T. C. Bruice and A. F. Hegarty, *Proc. Natl. Acad. Sci. U. S.*, **65**, 805 (1970).

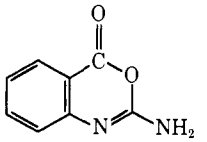
(5) D. Y. Curtin and L. L. Miller, *Tetrahedron Lett.*, 1869 (1965).

rangement of isoimides (**4**) is much akin to the N → O acyl transfer reaction which yields *N*-acylureas as by-products in the carbodiimide reaction (**5**).[6]



4 **5**

2-Amino-4,5-benzo-6-oxo-1,3-oxazine (**6**) is a suitable



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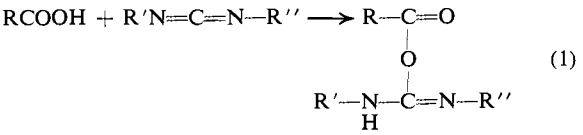
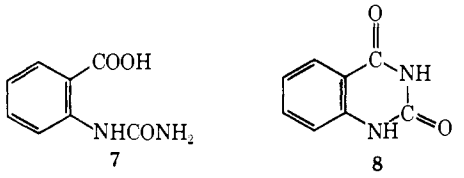
model for both the hypothetical structure of **1** as well as that of a carboxylic acid adduct to a carbodiimide (eq 1). Investigation of the reactions of **6** with nucleo-



(1)

philes should, therefore, shed light upon the mechanism of acyl group transfer in the carbodiimide reaction and conceivably CO_2 transfer in the biotin–carboxylase reaction. In this paper we describe the acid–base equilibria and isomeric forms of **6** in aqueous solution and discuss the kinetics and mechanism of the hydrolysis of **6** to 2-ureidobenzoic acid (**7**), and present some preliminary observations on the conversion of **7** to 2,4-(1*H*,3*H*)-quinazolinedione (**8**).



7 **8**

Experimental Section

Materials. 2-Amino-4,5-benzo-6-oxo-1,3-oxazine (6). A suspension of 21.2 g of cyanogen bromide (0.20 mol) was vigorously stirred at 5° in 100 ml of water. To this was added 15.9 g of sodium anthranilate (0.10 mol) in 100 ml of water over a period of 15 min. The precipitated product was stirred for a further hour, filtered off, and washed with water. The benzoxazine was recrystallized from absolute dioxane (care being taken to avoid decomposition resulting from prolonged heating) to give 12.6 g (78%), mp 212° dec (lit.[7] mp 210–212°).

2-Ureidobenzoic Acid (7). Anthranilic acid (13.7 g, 0.10 mol) was dissolved in 150 ml of 50:50 glacial acetic acid–water and cooled to 0°. The solution was treated dropwise with an equimolar quantity (8.1 g) of potassium isocyanate dissolved in 30 ml of water. The urea (14.0 g, 85%), which precipitated after 1 hr, was filtered and washed thoroughly with water. On recrystallization from methanol (with the addition of decolorizing charcoal and maintaining the temperature <50°) the *o*-ureidobenzoic acid had mp 170–171° (when heated rapidly to 160°; on melting, the material is converted to 2,4-(1*H*,3*H*)-quinazolinedione (**8**), which remelts at *ca.* 320°); lit.[8] mp 170–171°. 2,4-(1*H*,3*H*)-Quinazolinedione was synthesized by the method of Lange and Sheibley[9] and had mp 350–352° (lit.[10] 352–353° dec).

Kinetic Measurements. All kinetic experiments and pK_a determinations reported here were carried out in aqueous solution at 30° with μ = 1.0 (KCl). The rate of formation of **7** from **6** was followed by recording the decrease in optical density at 265 nm over all the pH range studied except below pH 1.5 where it was found that the decrease at 229 nm was more conveniently followed. Since the ring-opening reaction is strongly subject to buffer catalysis, determination of hydrolytic reaction rates by the usual methods of extrapolation to zero buffer concentration at constant pH and ionic strength gave at best tenuous results (since the extrapolated values were close to the origin). A technique was therefore used in which pH was maintained by the addition of alkali controlled by a pH-stat. A 25-ml volume thermostated cell (3-cm path length) was placed in the sample chamber of a Cary 15 spectrophotometer.[11] The contents of the cell were stirred magnetically, the drive motor being located in the upper portion of the reference chamber. Into the cell, but above the light beam, dipped a thermometer (which was removed to allow introduction of the substrate), a metrohm EA 125 U pH electrode (which both recorded the pH in the solution and controlled the addition of base), and a capillary Teflon tube (through which the base was added). The Radiometer pH-stat assembly consisted of a Type PHM 26 pH meter, a Titrator 11 Type TTT 11b, Titrograph Type SBR 2c, and an Autoburette Type ABU 1C. Typically, 25 ml of 1.0 *M* potassium chloride solution was added to the cell; the pH was then adjusted to and maintained at the desired pH by the pH stat assembly. The substrate was then added from a concentrated solution (*ca.* 1 mg/ml) in dioxane or acetonitrile. Care had to be exercised in using dioxane since the ring opening was catalyzed by the presence of this solvent (Matheson Coleman and Bell Spectroquality dioxane, used without further purification); at pH 10 the observed rate constant was approximately an order of magnitude greater in the presence of 4% dioxane. No such acceleration was observed with acetonitrile. To obviate such complicating factors, therefore, the organic component was maintained at a low value, usually *ca.* 0.1% of total solvent concentration. At this low concentration the observed rate constants were independent of the nature of the organic component. On completion of the kinetic run, the nature of the product formed was determined by a spectral method (see below) in the same cell.

Except in a limited number of cases at low pH (these are noted in the text) the observed OD *vs.* time curves were strictly first order and stable and reproducible infinity values were obtained. The values of the pseudo-first-order rate constants were calculated either graphically or by means of an Olivetti-Underwood Programa 101 computer using a weighted least-squares analysis (both methods giving equivalent results). For some of the very slowest reactions (pseudo-first-order rate constants $< 10^{-5}$ sec^{-1}), the OD change was measured to *ca.* three half-lives and the rate constants determined by the method of Guggenheim.

pK_a Determinations. The acid dissociation constants of **7** and **8** were determined at 30° and μ = 1.0 (KCl) using the same spectrophotometric titration cell described above. A known quantity of **7** was added to the cell and the pH was adjusted to *ca.* 2. Aliquots of 1.0 *N* potassium hydroxide were added and the change in OD at 320 nm recorded at each 0.1 pH interval. A plot of OD *vs.* pH was then compared with a theoretical titration curve to give the pK_a of **7**. A plot of milliliters of base added *vs.* pH yielded the same value. The pK_a of **8** was determined similarly, the spectrometric determination being most conveniently carried out at 260 nm.

The measurement of the acid and base dissociation equilibria of **6** was complicated by the fact that at the pH values around the pK_a's the hydrolytic rate is close to maximal. The following techniques obviated errors due to concomitant hydrolysis. A 20-ml aliquot of a 1.0 *M* aqueous KCl solution was transferred to the titration cell and brought to the desired pH using the pH-stat. Exactly 0.10 ml of the organic solvent containing the substrate was added rapidly and the change in OD with time recorded for 2–3 min. Extrapolation of this OD *vs.* time plot to zero time (con-

(6) M. Smith, J. G. Moffatt, and Khorana, *J. Amer. Chem. Soc.*, **80**, 6204 (1958).

(7) K. Lempert and G. Doleschall, *Monatsh. Chem.*, **95**, 953 (1964).

(8) R. P. Staiger and E. C. Wagner, *J. Org. Chem.*, **18**, 427 (1953).

(9) N. A. Lange and F. E. Sheibley, "Organic Syntheses," Coll. Vol. II, Wiley, New York, N. Y., 1943, p 79.

(10) O. Seide, *Ann. Chem.*, **440**, 319 (1924).

(11) For further constructional details of the cell, see J. R. Maley and T. C. Bruice, *Anal. Biochem.*, **34**, 275 (1970).

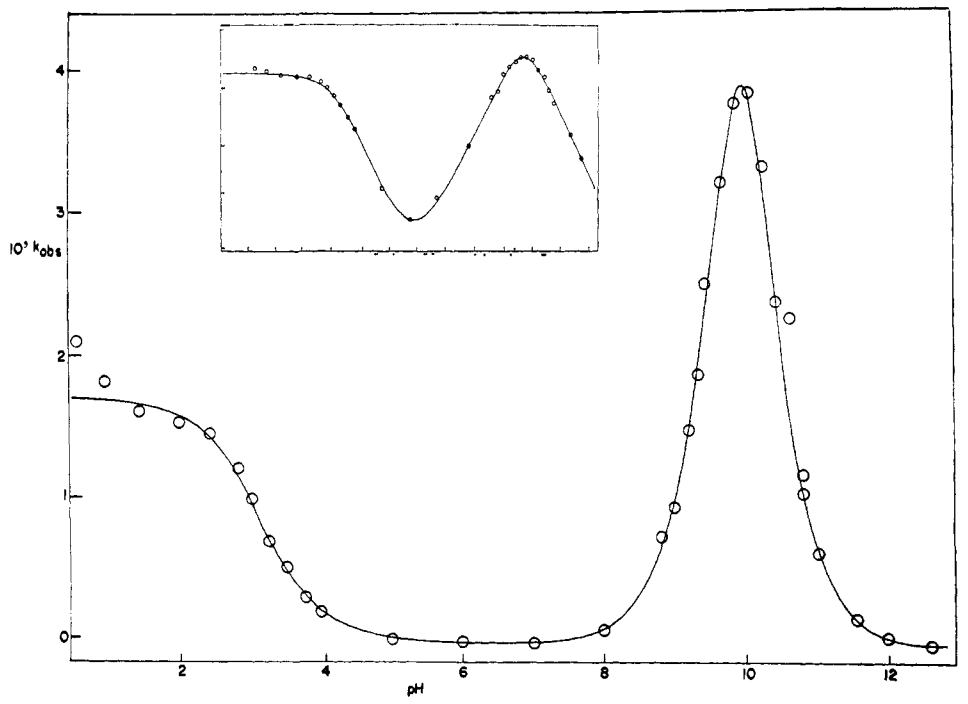


Figure 1. pH–rate profiles (k_{obsd} in sec^{-1}) for the hydrolysis of 2-amino-4,5-benzo-6-oxo-1,3-oxazine (**6**) (H_2O, μ = 1.0, 30°). Points are experimental and the curves generated from eq 8. Insert represents a plot of log k_{obsd} *vs.* pH.

veniently marked by the large change in OD on addition of the substrate) then gave the OD reading for the unhydrolyzed substrate at that pH. While maintaining the conditions rigorously the same, OD readings were obtained at various pH's (0.1 unit apart near the pK_a). Since the maximal rate of hydrolysis in any pH region did not excess 4×10^{-3} sec^{-1}, *i.e.*, minimal $t_{1/2}$ = 175 sec, and the equilibration time on the addition of substrate was at most 5 sec, the extrapolation involved in no case greater than 5% of the total change in OD on hydrolysis of **6**. Moreover the lower pK_a (3.10) was most conveniently measured at 340 nm. At this wavelength the absorption due to the reactive protonated species approaches zero. Therefore the larger OD readings (which of necessity involve more uncertain extrapolations) were obtained only when the hydrolytic rate was very slow. The OD *vs.* pH curves obtained by this method were reproducible and fitted precisely theoretical curves. The higher pK_a's (*ca.* 10) were also measured by this method, at two wavelengths, 280 and 340 nm. At 280 nm the hydrolytic product, **7**, has an absorption intermediate between the acid and base forms of the substrate, minimizing the OD change on reaction. The pK_a's calculated at both wavelengths were identical.

Product Analysis. Over all the pH range studied, the primary hydrolytic product was **7**. This was shown (a) by the identity of the spectrum of the reaction product (run immediately after a kinetic run) with that of an authentic sample of 2-ureidobenzoic acid and (b) by measuring the spectrum of the product at various pH's in the same reaction cell[11] on completion of hydrolysis. The pK_a obtained from these data was identical with that determined for 2-ureidobenzoic acid.

At low pH and again at high pH,[12] **7** slowly recyclized (but this time through attack by the terminal ureido nitrogen on the carboxyl group) to give **8**. This product was identified similarly (comparison of the spectra obtained at various pH's with those of authentic **8**).

In addition these transformations have been shown to occur in high yield on a preparative scale.[13] Thus, from the acid-catalyzed hydrolysis of **6** (1 g in 20 ml of H_2O), 79% of pure **7** was isolated; under neutral conditions in the same concentration range up to 71.5% yield was obtained.

Results

The rates of ring opening of 2-amino-4,5-benzo-6-oxo-1,3-oxazine (**6**) to give 2-ureidobenzoic acid (**7**) have been measured in aqueous solution at 30° (μ = 1.0) over the pH range 0.5–12.6. Two distinct regions in the pH profile are discernible: (a) a sigmoid-type dependence of k_{obsd} on pH at lower pH and (b) a bell-shaped region at high pH (Figure 1).

(a) The solid line, which best fits the experimental points at low pH in Figure 1, was derived from eq 2

$$k_{obsd} = k_1' \frac{a_H}{a_H + K_{a_1}} \qquad (2)$$

which is in accord with the kinetic scheme of eq 3. The

$$\mathrm{A} \underset{-H^+}{\overset{-H^+}{\rightleftharpoons}} \mathrm{B};\ K_{a_1} = \frac{[B][H^+]}{[A]} \qquad (3)$$

$$\mathrm{A} \xrightarrow{k_1'} \text{products}$$

constants which provide the best fit of the curve are $k_1' = 1.70 \times 10^{-3}$ sec^{-1} and $pK_{a_1} = 3.10$.

In addition, at very low pH (<1.0) the observed rate constants appear to rise again (Figure 1) with decreasing pH (at 0.5, the apparent observed rate constant is *ca.* 10% above the plateau value of 1.7×10^{-3} sec^{-1}). We prefer to attribute this to an artifact of the experimental method, rather than to the intervention of an additional mode (*e.g.*, specific acid catalysis) of reaction of the oxazine. At pH 1.0, the acid-catalyzed recyclization of **7** to **8** occurs at a rate ~6% of the initial ring-opening reaction. The observed rate constants for the cyclization of **7** → **8** under the same conditions are listed in Table I. Therefore, considerable recyclization was occurring at the lowest pH's before the initial ring opening of the oxazine was complete, leading to the erroneously high observed rate constants.

The solvent deuterium isotope effect in the ring opening was also measured in this pH region. The results obtained (Table II), give a $(k_1')_{D_2O}$ value of 8.5 ×

(12) A. F. Hegarty and T. C. Bruice, *J. Amer. Chem. Soc.*, **91**, 4924 (1969).

(13) G. Doleschall and K. Lempert, *Monatsh. Chem.*, **95**, 1068 (1964).

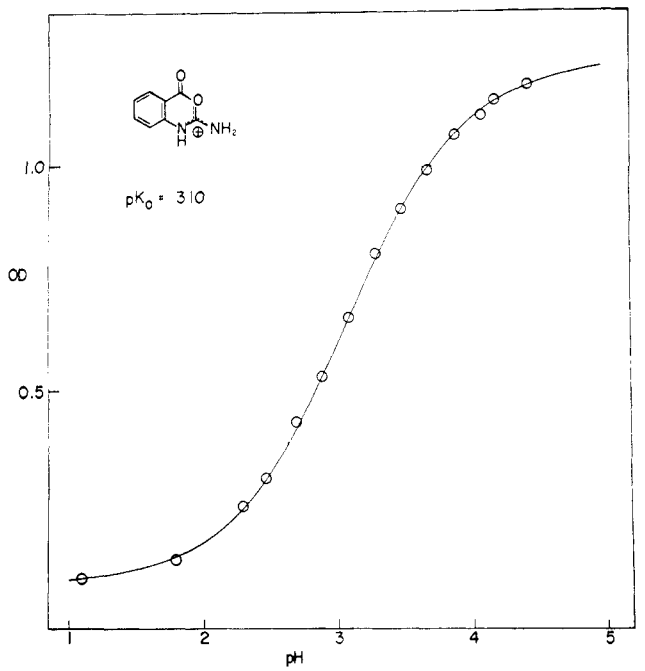


Figure 2. Spectrophotometric titration of **6** in the pH range 1.0–4.5 (H_2O, μ = 1.0, 30° at 340 nm). Points are experimental and the curve theoretical.

10^{-4} sec^{-1}, which indicates a solvent isotope effect $(k_1')_{H_2O}/(k_1')_{D_2O} = 2.0$ for the hydrolysis of species A.

The oxazine (**6**) also showed a distinct spectral change on pH variation in this region. This change was most readily followed at 340 nm; the observed OD *vs.* pH

Table I. Observed First-Order Rate Constants for the Cyclization of 2-Ureidobenzoic Acid to 2,4-(1*H*,3*H*)-Quinazolinedione[a]

pH	$10^4 k_{obsd}$, sec^{-1}
0.00	2.37
0.40	1.35
1.00	0.94
1.56	0.42
2.12	0.33

[a] At 30°; μ = 1.0.

Table II. Observed Pseudo-First-Order Rate Constants for Hydrolysis of 2-Amino-4,5-benzo-6-oxo-1,3-oxazine (**6**) in D_2O and H_2O[a]

pH (or pD[b])	$10^4(k_{obsd})_{H_2O}$[c]	$10^4(k_{obsd})_{D_2O}$
1.32	16.4	8.5
1.77	15.3	8.4
2.72	8.7	5.9

[a] At 30°; μ = 1.0. [b] Calculated using the relation pH + 0.38 = pD [T. H. Fife and T. C. Bruice, *J. Phys. Chem.*, **65**, 1079 (1961)]. [c] Sec^{-1}; calculated from eq 1.

values are plotted in Figure 2. In each case correction has been made in the OD reading for the concomitant hydrolysis. The line drawn in Figure 2 represents the best fit of the data with a theroetical OD–pH curve for a substrate with pK_a = 3.10. This spectrophotometrically determined pK is identical with the value of pK_{a_1} determined kinetically.

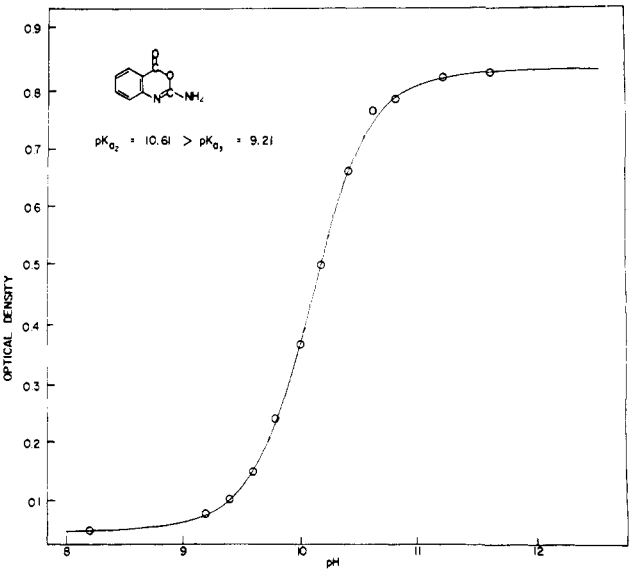


Figure 3. Spectrophotometric titration of **6** between pH 8 and 12 (H_2O, μ = 1.0, 30° at 280 nm). Points are experimental and curve theoretical.

(b) From Figure 1 it can be seen that in the pH region 7–12, k_{obsd} first increases with pH, passes through a maximal value at pH 9.91, then decreases rapidly. At pH 13 the hydrolysis is extremely slow ($k_{obsd} < 10^{-5}$ sec^{-1}). Analysis of this "bell-shaped" pH–rate profile was attempted by the method of Alberty and Massy,[14] who have shown that the equation which describes such a curve (eq 4) is best treated when the pK_a's are close

$$k_{obsd} = \frac{k_\gamma K_{a_1}' a_H}{K_{a_1}' K_{a_2}' + K_{a_1}' a_H + a_H^2} \quad (4)$$

together (K_{a_1}' refers to the ascending and K_{a_2}' to the descending legs of the bell) by use of the subsidiary eq 5 and 6. Although the pH (pH_{max}) where k_{obsd} is

$$pH_{max} = {}^1/_2(pK_{a_1}' + pK_{a_2}') \quad (5)$$

$$a_{H_1} + a_{H_2} = K_{a_1}' + 4a_{H_{max}} \quad (6)$$

maximal could be accurately calculated as 9.91 from the data, exact values of pK_{a_1}' and pK_{a_2}', on the contrary, could not. This was because the sum of the hydrogen-ion activities at one-half the height of the profile on the acid and base side ($a_{H_1} + a_{H_2}$) was close in magnitude to (actually greater than) $4a_{H_{max}}$. An estimate was made using the titration data, giving pK_{a_1}' = 10.51 and pK_{a_2}' = 9.31; *i.e.*, the apparent pK_a's have "crossed over," the limb at low pH being due to the higher pK_a. A satisfactory fit of the experimental data was obtained using these pK_a's, but similarly any pK_a's (related by eq 5) within ±0.2 unit of these values gave apparently an equally good fit. Without additional data as to the equilibria involved it is impossible from the kinetic data to determine with better precision the apparent equilibrium constants.

The oxazine (**6**) also showed a distinct spectral change in the ultraviolet between pH 7 and 12 (Figure 3). Correcting for concomitant hydrolysis, OD *vs.* pH titration curves were obtained at several wavelengths

(14) R. A. Alberty and V. Massy, *Biochim. Biophys. Acta*, 13, 347 (1954).

(see Experimental Section). Data at 280 nm are shown in Figure 4. It is seen that the experimental data do not describe the theoretical curve (such as that shown in Figure 2) expected for a single monoionizable group (*i.e.*, the OD points apparently first rise too sharply and then come to a constant value too quickly as the pH is increased). Instead a combination of 2 pK_a's is involved in this region. The individual pK_a's were determined by employing the eq 7 of Roth and Bunnett.[15] At any pH

$$OD_{a_H} = \frac{OD_B a_H^2 + OD_C K_{a_2} a_H + OD_D K_{a_2} K_{a_3}}{a_H^2 + K_{a_2} a_H + K_{a_2} K_{a_3}} \quad (7)$$

where B and D are the species in acidic and basic solution and OD_C is the absorption of the intermediate species C and OD_{a_H} is the observed optical density at a given pH. A theoretical fit for the experimental spectral data using this equation[16] gave pK_{a_2} = 10.61, pK_{a_3} = 9.21 at both 280 nm (see Figure 3) when OD_B = 0.05, OD_C (calcd) = 0.05, OD_D = 0.83, and at 340 nm where OD_B = 0.01, OD_C = 1.00, and OD_D = 0.465. It is clear that these pK's may also be used to fit the kinetic data to the bell-shaped curve. In fact, there is only one set of pK's which fit both the spectrophotometric and kinetic data. The bell-shaped curve determines $^1/_2$(pK_{a_2} + pK_{a_3}) as 9.91, while the spectrophotometric titration is very sensitive to the "spread" of the pK's from this central point.

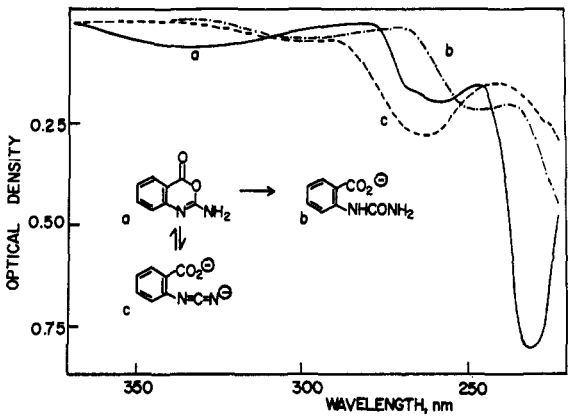


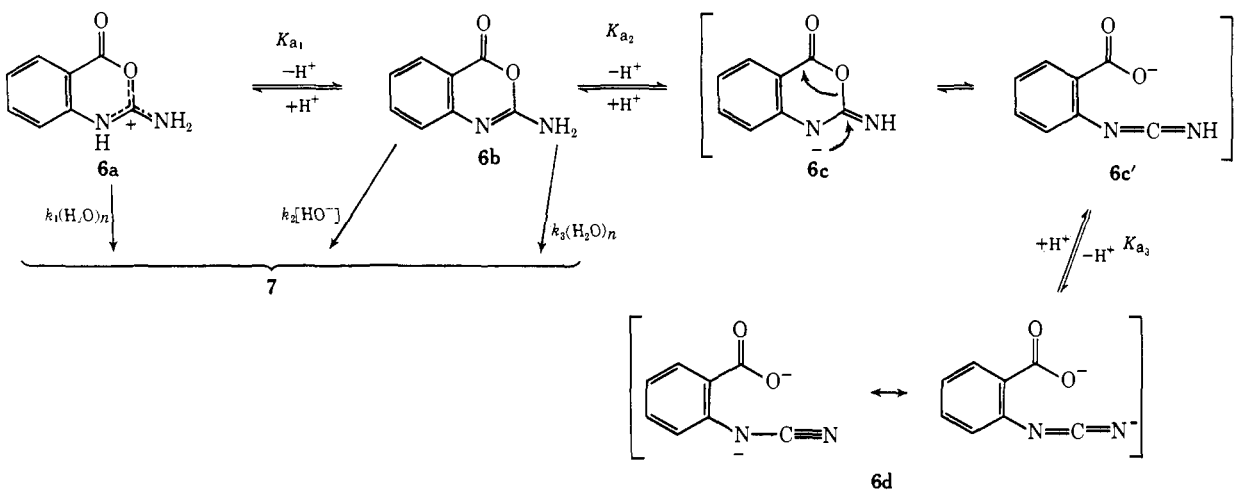
Figure 4. Ultraviolet spectra of **6** at pH 8 (species a), pH 11 (species c), and the product formed on hydrolysis of **6** (species b).

K_{a_2} = 2.43 × 10^{-11} M; K_{a_3} = 6.17 × 10^{-10} M, and

$$k_{obsd} = \frac{k_1' a_H}{a_H + K_{a_1}} + \frac{k_2' K_{a_2} a_H}{K_{a_2} K_{a_3} + K_{a_2} a_H + a_H^2} + k_3'$$

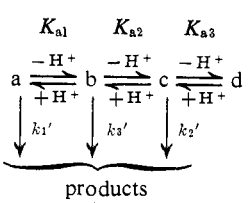
The line drawn in the plot of log k_{obsd} *vs.* pH (Figure 1) was calculated using this equation. The term k_3' is significant only in the pH range of 5–7 where k_{obsd} is minimal.

Scheme II[a]



[a] k_1 (n = 1) = 3.0 × 10^{-5} M^{-1} sec^{-1}; k_2 = 51 M^{-1} sec^{-1}; k_3 (n = 1) = 4.5 × 10^{-8} M^{-1} sec^{-1}; K_{a_1}, K_{a_2}, and K_{a_3} have the values given in eq 8.

A kinetic scheme, therefore, may be written for the hydrolysis of **6** which is consistent with the experimental data



(8)

where k_1' = 1.70 × 10^{-3} sec^{-1}; k_2' = 3.10 × 10^{-2} sec^{-1}; k_3' = 2.4 × 10^{-6} sec^{-1}; K_{a_1} = 7.96 × 10^{-4} M;

(15) B. Roth and J. F. Bunnett, *J. Amer. Chem. Soc.*, 87, 334 (1965).
(16) A program written for the Olivetti-Underwood Programa 101 by Dr. D. Tanner, formerly of this laboratory, was used.

Discussion

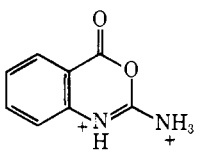
The k_{obsd} *vs.* pH profile for the conversion of **6** to **7** in Figure 1 has been shown to be consistent with the empirical expression in eq 8. Rather than a number of other kinetically indistinguishable mechanisms which would also fit the observed data, the reactant species favored are present in Scheme II, where **6a** is associated with a in eq 8, **6b** with b, etc.

The observed pH profile in Figure 1, which shows a sigmoid-type curve at low pH and a "bell"-shaped region at high pH, does not definitely establish that three acid ionizations are involved (any of the steps, of course, may be addition of hydroxide rather than loss of a proton). One or possibly both of the limbs of the bell could be due to a combination of kinetic terms.

However, each of the pK_{app}'s required by the kinetic scheme is associated with a spectral change in the substrate. The three pK's calculated from the spectral data are identical with those required to fit the kinetic data. In addition, no build-up of intermediate products was observed (uv repetitive scans of the conversion of **6** to **7** *vs.* time had tight isosbestic points). Therefore, a minimum of four substrate species related by three acid–base equilibria are required.

The observed kinetic scheme of eq 8 implies the unusual situation that $pK_{a_2} > pK_{a_3}$. Thus the substrate, in going from a → d as the pH is raised, appears to lose the third proton *more* readily than the second. Further evidence that the equivalent of two protons is lost between b (which is the major species of **6** at pH 7) and d was obtained by titration of the substrate with potassium hydroxide or hydrochloric acid. If the titration is carried out rapidly, then the period when the solution is *ca.* pH 9.9, and thus liable to be hydrolyzed at its maximum rate, is reduced. Several such determinations, starting either with d or with b, gave an average value of 1.8 equiv (maximum value 1.9 equiv) of base (or acid) per mole of substrate used. Moreover the equilibrium b → d is reversible. This was shown by recording the spectrum of **6** at pH 7 (see Figure 4); at this pH the hydrolysis of **6** is negligible during the time taken to record the spectrum. The pH of the solution was then raised rapidly to 12 (where hydrolysis is again slow) and the spectrum of d recorded (Figure 4). On reacidification to pH 7 the observed spectrum of **6** was the same as that initially observed. Several such acidification–basification cycles could be carried out before appreciable hydrolysis of **6** obscured the spectra.

A fifth species, **6e**, could also be considered in the kinetic scheme. If this were a in eq 8 then b, c, and d



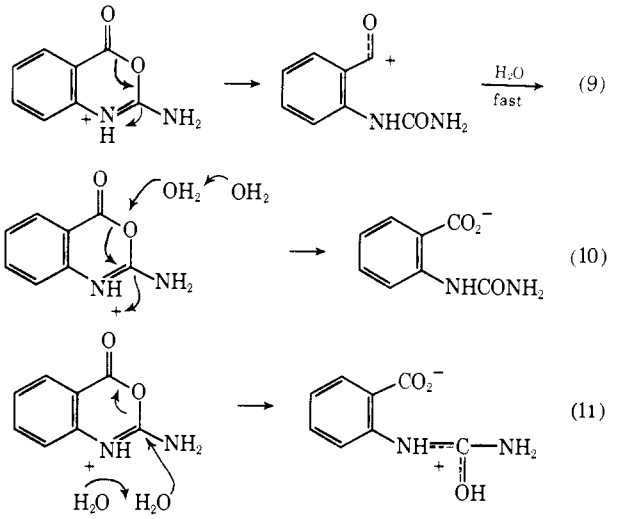
6e

would be the monoprotonated, neutral, and monoanion, respectively. Such a scheme would eliminate the involvement of dianionic species and might make the "crossed" pK's appear more reasonable. Thus the monoanionic species (**6c**) is stabilized by the possibility of several resonance forms; it is conceivable, although unlikely, that this stabilization might be sufficient that loss of a proton from the neutral species **6b** occurs more readily, *i.e.*, at lower pH, than from **6a**. The necessity for the intervention of **6e** can, however, be eliminated by the following considerations. (1) For the condition that a = **6e**, the pK_a of **1e** would be 3.10. This is unlikely since guanidine, in which the basic site is analogous to **6** with an amino replacing the acyl group, is protonated very readily (pK_a of the conjugate acid = 13.6 at 25°), but addition of a second proton is very difficult; the second pK_a of guanidine has been estimated[17] as −11. (2) Treatment of **6** with an excess of dry HCl in dioxane solvent yields a mono-, rather than dihydrochloride of **7**.

(17) G. Williams and M. L. Hardy, *J. Chem. Soc.*, 2560 (1953).

The observed kinetic scheme of eq 8 implies that the hydrolysis of the substrate is minimal at high pH where d is essentially the sole species present. This suggested that species d could be isolated and characterized. This was in fact most readily done by dissolving **6** in ethanol and rapidly adding an excess of sodium ethoxide. A sodium salt immediately precipitated which could be recrystallized from ethanol–ether. Analysis of an aqueous solution of the salt by atomic absorption spectroscopy showed that it contained 2.0 mol of sodium per mole of **6**, suggesting that d is in fact a dianionic species. Use of an equimolar amount of sodium ethoxide resulted in the precipitation of a small amount of the dianion, rather than of a monoanionic species. The fact that there are only two protons in **6** outside the aromatic ring and available for abstraction limited the possible structures of d. Its ultraviolet spectrum (Figure 3) showed a long-wavelength absorption almost identical with that of 2-ureidobenzoate, suggesting the presence of a free carboxy anion. The ir spectrum of the disodium salt of d had a high-intensity absorption at 2110 cm^{-1}; this is in the region normally observed for carbodiimides or nitriles,[18] thus lending strong support to the assignment of the open-chain formulation of the dianion (**6d**) to the species d.

With the tentative assignment of the species involved (in Scheme II), the mechanism can be considered in more detail. Three mechanisms may be considered for the spontaneous rate of hydrolysis of **6a**, see eq 9, 10, and 11. The reactions of eq 10 or 11 which involve



(9)

(10)

(11)

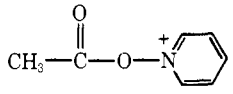
rate-determining (water assisted) attack by water on the protonated species are favored over eq 9[19] by the solvent kinetic isotope effect of 2.0. Distinction between 10 and 11, which is essentially between the two possible electrophilic centers in **6a**, is more difficult. Attack at either position leads to the same product (*i.e.*, **7**). However, eq 10 is the more likely path for the following reasons: (a) the leaving group is of lower pK_a [the pK_a of phenylurea is −0.3 at 25°,[20] while the pK of the conjugate acid of the leaving group in 5 is 3.36 (30°, μ = 1.0)]. (b) With nucleophiles other than water or

(18) L. J. Bellamy, "The Infra-red Spectra of Complex Molecules," Methuen, London, 1962, pp 263–267.

(19) G. Doleschall and K. Lempert, *Acta Chim.* (*Budapest*), **40**, 235 (1964).

(20) D. D. Perrin, "Dissociation Constants of Organic Bases in Aqueous Solution," Butterworths, London, 1959, p 451.

hydroxide ion, no such ambiguity arises and the products formed by routes 10 and 11 are readily distinguished. With amines as nucleophiles, *o*-ureido amides are formed by attack at the lactone carbonyl group in **6a**[21] (which is analogous to eq 10), rather than substituted guanidines. Assuming mechanism 10, the value calculated for the second-order rate constant for reaction of **6a** with water is $3.0 \times 10^{-5}\ M^{-1}\ \text{sec}^{-1}$. This value is close to that observed by Jencks and Gilchrist[22] for hydrolysis of 1-acetoxy-4-methoxypyridinium perchlorate **9** (*viz.* $2.2 \times 10^{-4}\ M^{-1}\ \text{sec}^{-1}$); with other nucleophilic agents, such as amines, this parallel is also noted.[21]



9

Spontaneous reaction of species c (with water) is kinetically equivalent to reaction of b with hydroxide ion (Scheme II). If reaction with water is being observed, then c must be a particularly reactive species, since the second-order rate constant calculated ($5.2 \times 10^{-4}\ M^{-1}\ \text{sec}^{-1}$) is 18-fold greater than that for reaction of the same nucleophile with protonated **6**. Neither the open (**6c′**) or cyclic (**6c**) forms of the anion could explain this apparent difference. The *o*-carboxyphenylcarbodiimide **6c′** would be expected by comparison with other carbodiimides to have a relatively slow rate of spontaneous hydrolysis. Thus $t_{1/2}$ for the hydroxide-catalyzed hydrolysis of symmetrical diphenylcarbodiimide is 18 min at 20° in 75% THF containing 0.02 *M* NaOH;[23] even this value is considerably slower than that calculated for **6c′** with water. Moreover **6c′** is in equilibrium with a tautomeric form, *N*-cyanoanthranilic acid; with monosubstituted carbodiimides this is the favored isomer.[24] On this basis it is unlikely that the large rate constant observed could be attributed to the spontaneous hydrolysis of **6c**. Even under optimum conditions, the concentration of **6c** never exceeds 10% of the total **6** present (due to $pK_{a_2} > pK_{a_3}$); moreover when the mole fraction of **6c** is maximal (at pH 9.9) hydrolysis of **6** is also maximized. Therefore exact structural assignment is difficult.

Assuming that the equivalent reaction of **6b** with hydroxide ion is operative a second-order rate constant ($k_3' = 49\ M^{-1}\ \text{sec}^{-1}$ can be calculated ($k_3' = k_3K_2/K_w$). This value is not unreasonable when considered together with data for the reaction of other nucleophiles with **6b**;[7] the point for HO^- lies between two and three orders of magnitude below the Brønsted plot for the amines. Although the negative deviation shown by HO^- from such Brønsted plots with simple ester substrates varies, this is not an atypical value. Note that Scheme II therefore demands that species **6c** and **6d** do not undergo nucleophilic attack. With nucleophiles other than lyate species where a more definite distinction between the reactive species is possible, a similar scheme is required (*i.e.*, reaction is only detected with **6a** and **6b**).[21]

Acknowledgment. This work was supported by grants from the National Science Foundation and the National Institutes of Health.

(21) A. F. Hegarty and T. C. Bruice, *J. Amer. Chem. Soc.*, **92**, 6568 (1970).
(22) W. P. Jencks and M. Gilchrist, *ibid.*, **90**, 2622 (1968).
(23) S. Hunig, H. Lehmann, and G. Grimmer, *Justus Liebigs Ann. Chem.*, **579**, 87 (1953).
(24) F. Kurzer and K. Douraghi-Zadeh, *Chem. Rev.*, **67**, 107 (1967).