Direct Observation of a Tetrahedral Intermediate during Amidine Hydrolysis[1]

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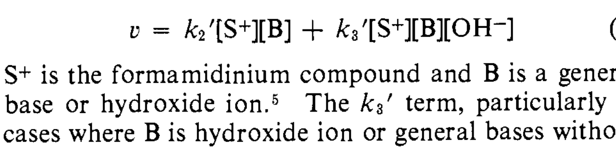
Abstract: The hydrolysis of the formamidinium compound, 1,3-diphenyl-2-imidazolinium chloride (DPIC), to form N-(2-anilinoethyl)formanilide (AEF) has been followed spectrophotometrically in aqueous potassium hydroxide solutions at 25.0°, ionic strength 1.0 *M*. When followed at 255 mμ at pH values above 11.6, there is a rapid initial increase in absorbance followed by a slower first-order decrease, demonstrating that an intermediate accumulates during the course of the reaction. The results are quantitatively accounted for by a mechanism in which a tetrahedral intermediate is formed by the attack of hydroxide ion on DPIC and accumulates during the initial rapid phase of the reaction. The intermediate breaks down through its anionic conjugate base to form AEF. Pseudo-first-order rate constants for the slower phase of the reaction show a nearly second-order dependence on hydroxide ion activity at lower pH values, where the intermediate accumulates to a small extent. As the pH increases the intermediate accumulates in larger amounts, and the dependence of the rate constants on hydroxide ion activity decreases, with the rates becoming almost pH independent near pH 13. This tendency of the rate constants to become pH independent at high pH is accounted for by the ionization of the intermediate with a pK_a value of 12.75. The ultraviolet spectrum of the intermediate has a peak at 252 ± 2 mμ with an extinction coefficient of approximately 2.2×10^4 M^{-1} cm^{-1} and a weak shoulder in the 290–300 mμ range. The possibility that the intermediate is the ylide formed from the ionization of the C-2 proton from DPIC is considered to be ruled out both by the ultraviolet spectrum of the intermediate and by the requirement for a pK_a value of 12.3 for ylide formation which appears to be unreasonably low. These experiments appear to represent the first clear example reported of the direct observation of a tetrahedral intermediate at the acyl level of oxidation during the course of a transfer or hydrolysis reaction.

Central to the understanding of the mechanisms of acyl transfer reactions is the problem of whether or not these reactions proceed through tetrahedral addition intermediates. Tetrahedral intermediates are readily observed during certain nucleophilic reactions of aldehydes and ketones, and the stability of hydrates of some of these compounds is well known. At the acyl level of oxidation, kinetic and exchange experiments provide strong evidence for the existence of tetrahedral intermediates on some of these reaction pathways, but the direct observation of a tetrahedral intermediate at the acyl level of oxidation during the course of transfer or hydrolysis reactions has not been previously reported.[2,3] The direct observation of an intermediate during the course of a reaction provides obvious advantages for the elucidation of the reaction mechanism. Even if it is possible to demonstrate by kinetic methods that an intermediate is present under steady state conditions, it may not be possible to determine whether the formation or breakdown of the intermediate is rate determining under given conditions. Furthermore, the direct observation of an intermediate may make it possible to measure individual rate constants both for its formation and breakdown, which is not possible under steady state conditions.

A study of the mechanisms of hydrolysis of two formamidinium compounds 1,3-diphenyl-2-imidazolinium chloride (DPIC) and N^{5,10}-methenyltetrahydrofolic acid has recently been reported.[4] Both compounds appear to hydrolyze by mechanisms which involve the rate-determining general acid–base catalyzed decomposition of tetrahedral addition intermediates at low buffer concentration (eq 1). There are two lines of evidence for the presence of these intermediates in the previous work. First, the hydrolysis of both compounds below pH 11 follows the rate law of eq 2 where



(1)

$$v = k_2'[S^+][B] + k_3'[S^+][B][OH^-] \qquad (2)$$

S^+ is the formamidinium compound and B is a general base or hydroxide ion.[5] The k_3' term, particularly in cases where B is hydroxide ion or general bases without ionizable protons, indicates that there are at least two steps on the reaction pathway and that a step following the formation of an intermediate is rate determining. Second, there is a change in rate law for N^{5,10}-methenyltetrahydrofolate hydrolysis with varying buffer concentrations, requiring a change in rate-determining step.

The previous work was performed in the pH range 7–11, and no evidence was obtained for the accumu-

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[1] Publication 504 from the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities. Supported by grants from the U. S. Public Health Service (AM-4501 and AM-3564).

[2] For recent reviews see: W. P. Jencks, *Progr. Phys. Org. Chem.*, 2, 63 (1964); T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. I, W. A. Benjamin, Inc., New York, N. Y., 1966; S. L. Johnson, *Advan. Phys. Org. Chem.*, **5**, 237 (1967); W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill Book Co., Inc., New York, N. Y., 1969.

[3] A possible exception to this statement is discussed below. See M. I. Vinnik and Y. V. Moiseyev, *Tetrahedron*, **19**, 1441 (1963).

[4] (a) D. R. Robinson and W. P. Jencks, *J. Amer. Chem. Soc.*, **89**, 7088 (1967); (b) **89**, 7098 (1967).

[5] The symbols [H$^+$] and [OH$^-$] indicate the activity of these species, based on pH measurements.

lation of the intermediate under these conditions. We report here the results of an investigation of the hydrolysis of DPIC under more alkaline conditions where the accumulation of an intermediate is clearly observed spectrophotometrically. These results appear to represent the first direct observation of a tetrahedral intermediate at the acyl level of oxidation during the course of a hydrolysis or transfer reaction. A preliminary report of this work has appeared.[6]

Experimental Section

The compound N-(2-anilinoethyl)formanilide (AEF) was prepared by the method of Zienty,[7] as previously described,[4a] and was recrystallized from ethanol, mp 62°. 1,3-Diphenyl-2-imidazolinium chloride (DPIC) was obtained by dissolving AEF in 0.02 *N* HCl with warming to *ca.* 50°, and allowing the solution to stand at room temperature for at least 0.5 hr. The anilide is completely converted to DPIC under these conditions and the ultraviolet spectrum of this solution remains stable for over 24 hr at room temperature. The product of the alkaline hydrolysis of DPIC has been well documented to be AEF[4,8] and was confirmed in this work by ultraviolet spectroscopy. Inorganic compounds and disodium EDTA were reagent grade materials and were used without further purification. Glass-distilled water was used throughout.

All experiments reported here were performed in aqueous potassium hydroxide solutions at 25.0° over the pH range 11.4–13.1. Ionic strength was maintained at 1.0 *M* with added potassium chloride, and approximately 1×10^{-4} *M* EDTA was present in all reactions. The initial concentrations of DPIC ranged from 5×10^{-5} to 7×10^{-5} *M*. All reactions were followed spectrophotometrically. Pseudo-first-order rate constants were calculated from plots of $\ln (A - A_\infty)$ against time on semilogarithmic graph paper and the relationship $k_{\text{obsd}} = 0.693/t_{1/2}$.

Measurements of pH were performed with Radiometer models PHM 4 and PHM 26 meters using Radiometer G 202 B glass electrodes. Solutions were thermostated at 25.0° during measurements. The meters were standardized at pH 7.00 and 10.00 with ordinary standard buffers. With this standardization calcium hydroxide solutions, saturated at 25.0°, gave readings of 12.42–12.46 (lit. 12.42).[9] A 0.010 *M* solution of trisodium phosphate gave a reading of 11.73 (lit. 11.72).[10] Hydroxide ion activity was defined by the relation, $\log a_{\text{OH}^-} = \text{pH} - 14.00$, where pH is the meter reading. Activity coefficients of hydroxide ion were calculated from these activity measurements and the known concentrations, and generally ranged from 0.65 to 0.72 over the pH range of these experiments without an obvious trend. This supports the assumption that the pH meter readings provide a reasonable estimate of, or are proportional to, hydroxide ion activity in these solutions.

Stopped-Flow Apparatus. Most of these experiments were performed with a stopped-flow apparatus which was kindly provided by Dr. W. P. Jencks. In later phases of this work a nearly identical apparatus was used in the author's laboratory and good agreement was obtained between the two instruments. Reactions are followed with a Gilford spectrophotometer Model 200 or 240, and recorded with a Tektronix Model 564 storage oscilloscope. Temperature was maintained at 25.0 ± 0.2° by circulating water through a brass block containing the driving syringes of the stopped-flow apparatus, and through thermospacers lining the spectrophotometer cell compartment which houses the mixing and observation chambers of the apparatus.

A typical oscilloscope tracing of the hydrolysis of DPIC, under conditions where the observable reaction follows pseudo-first-order kinetics throughout, is replotted on semilogarithmic coordinates in Figure 1. The tracings contain an initial horizontal portion of about 3-msec duration, which is interpreted as a period of continuous flow. This is followed by a first-order decay in absorbance after flow has been stopped. The relationship of the time scale of the reaction to the time of mixing ($t = 0$) is obtained by extrapolating the first-order plot back to the value of the absorbance at the initial part of the tracing. The point of intersection of the dashed lines is taken at 4.0 msec on the time scale of the first-order reaction, based on the measured dead time.

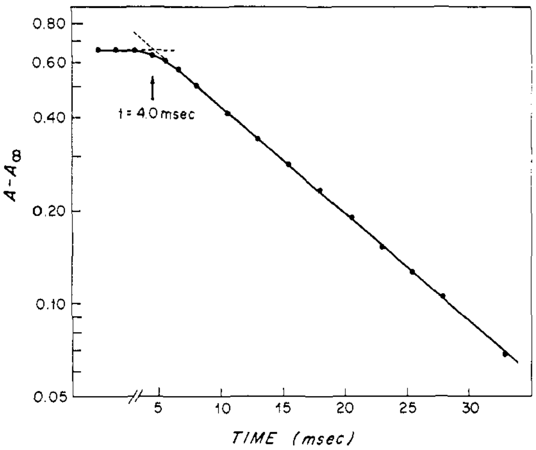

Figure 1. Replot of an oscilloscope trace of the hydrolysis of DPIC at pH 12.77 in potassium hydroxide solution at 25.0°, followed at 255 mμ. Under these conditions the initial rapid reaction is completed during the dead time of the instrument and the observed reaction is first order throughout. The intersection of the dashed lines is taken to be 4.0 msec on the time scale of the first-order reaction.

The dead time of the stopped-flow apparatus was determined from measurements of the hydrolysis of 2,4-dinitrophenylacetate in 0.50 *M* potassium hydroxide at 400 mμ, and from the hydrolysis of acetylimidazole in 0.20 and 0.50 *M* potassium hydroxide at 245 mμ. The temperature was 25.0° and ionic strength was maintained at 1.0 *M* with added potassium chloride. The dead time, t_d, was calculated from the first-order equation, $\ln [(A_\infty - A_0)/(A_\infty - A_t)]$ or $\ln [(A_0 - A_\infty)/(A_t - A_\infty)] = k_{\text{obsd}}t_d$. The terms A_0 and A_∞ are absorbance at zero and infinite time, A_t is the absorbance at the beginning of the oscilloscope tracing, and k_{obsd} is the observed pseudo-first-order rate constant for each reaction. Half-lives for these reactions ranged from 11.0 to 4.4 msec. The dead time of the instrument was found to be 4.0 ± 0.5 msec under the conditions of these experiments. This represents the time elapsed between the time of mixing ($t = 0$) and the first absorbance measurement which corresponds to the initial horizontal part of the tracings (Figure 1). These experiments also show that a further 1.0 msec elapses after the initial part of the tracings before the reactions become first order. Therefore, the age of the reaction mixtures at the time flow is stopped is 5.0 ± 0.5 msec.

Pseudo-first-order rate constants for the hydrolysis of acetylimidazole were an approximately linear function of hydroxide concentration over the range 0.05–0.50 *M*, and gave a second-order rate constant of 1.9×10^4 M^{-1} min^{-1}, based on hydroxide ion concentrations. A value of 1.9×10^4 M^{-1} min^{-1} has previously been reported for this reaction, based on hydroxide ion activity[11] at 25.0° and ionic strength 0.2 *M*.[12]

Calculations. The general equations describing two consecutive, reversible, first-order reactions (eq 3) are given by Frost and Pearson.[13] For the case in which the second step is irreversible ($k_{32} = 0$), the following equations are obtained, given that the initial conditions ($t = 0$) are $A_1 = A_0$ and $A_2 = A_3 = 0$.

$$A_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A_2 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} A_3 \qquad (3)$$

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(6) D. R. Robinson, *Tetrahedron Lett.*, 5007 (1968).

(7) F. B. Zienty, *J. Amer. Chem. Soc.*, **68**, 1388 (1946).

(8) M. May, T. J. Bardos, F. L. Barger, M. Lansford, J. M. Ravel, G. L. Sutherland, and W. Shive, *ibid.*, **73**, 3067 (1951); L. Jaenicke and E. Brode, *Ann.*, **624**, 120 (1959).

(9) R. G. Bates, "Determination of pH," John Wiley & Sons, Inc., New York, N. Y., 1964. The pH meter readings were unstable in calcium hydroxide solutions with a slow drift to higher pH values. The readings indicated were obtained 1–3 min after immersion of the electrodes.

(10) V. Gold, "pH Measurements," Methuen and Co., Ltd., London, 1956.

(11) W. P. Jencks, personal communication. Hydroxide ion activity was determined from pH measurements.

(12) W. P. Jencks and J. Carriuolo, *J. Biol. Chem.*, **234**, 1272 (1959).

(13) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd ed, John Wiley & Sons, Inc., New York, N. Y., 1961, pp 173–177.

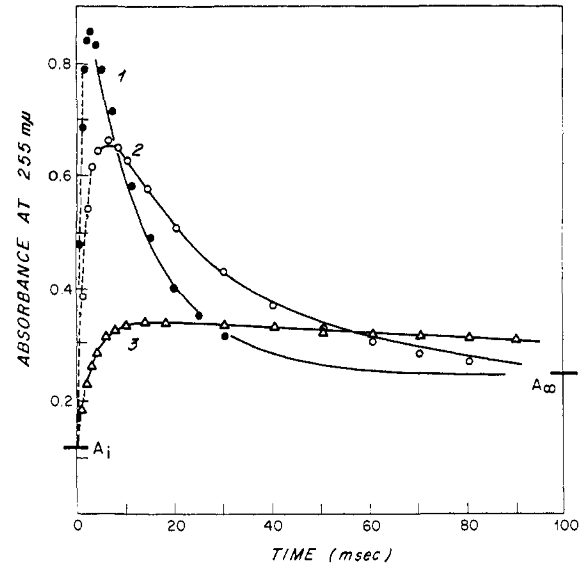


Figure 2. Hydrolysis of DPIC in potassium hydroxide solutions at 25.0°, ionic strength 1.0 *M*, followed at 255 mμ. The solid lines are observed absorbance and points (and dashed lines) are calculated as described in the text. The terms A_i and A_∞ are initial and infinite time absorbance values, respectively. The pH values are: curve 1, 12.77; curve 2, 12.38; and curve 3, 11.76.

$$A_1 = A^0\left[\frac{k_{12}(\lambda_2 - k_{23})}{\lambda_2(\lambda_2 - \lambda_3)}e^{-\lambda_2 t} + \frac{k_{12}(k_{23} - \lambda_3)}{\lambda_3(\lambda_2 - \lambda_3)}e^{-\lambda_3 t}\right]$$

$$A_2 = A^0\left[\frac{-k_{12}\lambda_2}{\lambda_2(\lambda_2 - \lambda_3)}e^{-\lambda_2 t} + \frac{k_{12}\lambda_3}{\lambda_3(\lambda_2 - \lambda_3)}e^{-\lambda_3 t}\right]$$

$$A_3 = A^0\left[\frac{k_{12}k_{23}}{\lambda_2\lambda_3} + \frac{k_{12}k_{23}}{\lambda_2(\lambda_2 - \lambda_3)}e^{-\lambda_2 t} - \frac{k_{12}k_{23}}{\lambda_3(\lambda_2 - \lambda_3)}e^{-\lambda_3 t}\right] \quad (4)$$

$$\lambda_2 = {}^1\!/_2[k_{12} + k_{21} + k_{23} + \sqrt{(k_{12} + k_{21} + k_{23})^2 - 4k_{12}k_{23}}]$$

$$\lambda_3 = {}^1\!/_2[k_{12} + k_{21} + k_{23} - \sqrt{(k_{12} + k_{21} + k_{23})^2 - 4k_{12}k_{23}}]$$

These equations are applied to the mechanisms of DPIC hydrolysis shown in eq 5 and 6, by describing these mechanisms in terms of pseudo-first-order rate constants at given pH values (Table I). The terms A_1, A_2, and A_3 are the concentrations of DPIC, total intermediate, and AEF, respectively. It is assumed that the ionization of the intermediate, step K_a in eq 5, occurs rapidly relative to the other steps. The equations were solved with the aid of a computer, giving the concentrations of DPIC, total intermediate, and AEF as a function of time at given pH values. Calculated absorbance curves and rate constants were obtained from this information as described in the Results. Values of the rate constants which gave the best fit to the experimental data were found by successive approximation.

Table I. Determination of Pseudo-First-Order Rate Constants for the Rate Equations from the Rate Constants of the Proposed Mechanisms of DPIC Hydrolysis

Eq 3	Eq 5	Eq 6
k_{12}	$k_1[OH^-]$	$k_1[OH^-]$
k_{21}	$k_{-1}[H^+]/([H^+] + K_a)$	k_{-1}
k_{23}	$k_2K_a/(K_a + [H^+])$	$k_2'[OH^-]$

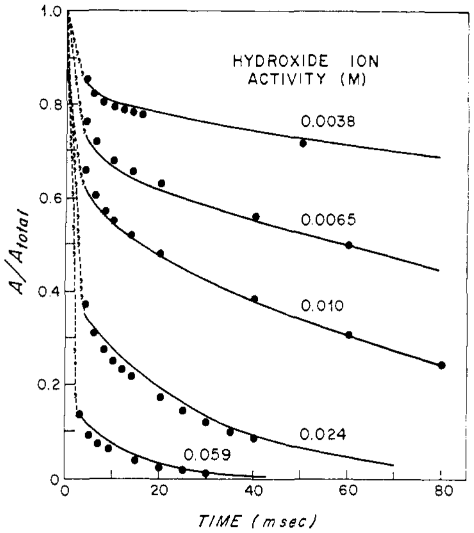
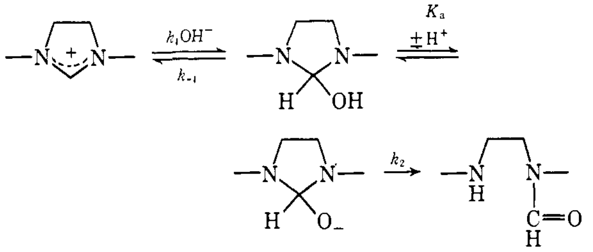


Figure 3. Hydrolysis of DPIC in potassium hydroxide solutions at 25.0°, followed at 320 mμ. A/A_{total} is the fraction of the initial absorbance. Solid lines are the observed absorbance tracings and points are calculated.

Results

The hydrolysis of DPIC was followed spectrophotometrically in a stopped-flow apparatus at 25.0° in aqueous potassium hydroxide solutions, and some typical results are shown in Figures 2 and 3. When the reaction is followed at 255 mμ at pH values above 11.6, there is a rapid increase in absorbance followed by a slower decrease. The extent of the initial increase in absorbance is greater with increasing pH, but this initial reaction is nearly completed during the dead time of the apparatus and cannot be characterized in detail. The subsequent decay of absorbance follows pseudo-first-order kinetics and the rate constants increase with increasing pH. At 320 mμ, near the absorption maximum of DPIC, there is a rapid partial loss of absorbance which is followed by a slower first-order decrease (Figure 3). The extent of the initial loss of absorbance increases with increasing pH, and corresponds to the initial increase in absorbance at 255 mμ.

The results are interpreted according to the mechanism of eq 5. A tetrahedral addition intermediate is formed from the attack of hydroxide ion on DPIC and accumulates during the initial rapid phase of the reactions. The intermediate then decomposes to form the product during the subsequent slower phase.



(5)

Pseudo-first-order rate constants for the slower phase of the reaction are shown as a function of hydroxide ion activity in Figure 4. At lower hydroxide ion activity, where the intermediate accumulates to a small extent, the rates are greater than first order with respect to hydroxide ion. This is expected on the basis of the previously reported work which showed that in the pH range 10–11 the rate is nearly entirely accounted for by a reaction which is second order in hydroxide ion. As hydroxide ion activity increases and large amounts of the intermediate accumulate, the dependence of the observed rate constants on hydroxide ion activity decreases and eventually the rates become nearly pH independent.

According to the mechanism of eq 5, the dependence of these rate constants on hydroxide ion activity should decrease with increasing pH as larger amounts of DPIC are converted to the intermediate during the initial rapid phase of the reaction. If the pK_a value of the intermediate were sufficiently higher than the pH range of these experiments, these rate constants should approach first order, but should not become less than first order, with respect to hydroxide ion at high pH. In this situation, the reaction could be described by eq 6 in which it is assumed that the conjugate base of the intermediate does not accumulate significantly in this pH range. The dashed line in Figure 4 is based on the scheme of eq 6 using the values of the rate constants

$$\text{DPIC} \underset{k_{-1}}{\overset{k_1[\text{OH}^-]}{\rightleftharpoons}} \text{TH} \xrightarrow{k_2''[\text{OH}^-]} \text{AEF} \qquad (6)$$

given in Table II. The large deviation of the observed rate constants below the dashed line in Figure 4 is attributed to the ionization of the intermediate. These results can be accounted for by the mechanism of eq 5, with a pK_a value of 12.75 for the intermediate.

Table II. Rate and Ionization Constants for DPIC Hydrolysis at 25.0°[a]

k_1	$1.6 \times 10^4\ M^{-1}\ \text{sec}^{-1}$
k_{-1}	$250\ \text{sec}^{-1}$
k_2	$180\ \text{sec}^{-1}$
K_a	$1.8 \times 10^{-13}\ M$
k_2'' [b]	$3.24 \times 10^3\ M^{-1}\ \text{sec}^{-1}$

[a] The terms are from eq 5 and 6. [b] $k_2'' = k_2(K_a/K_w)$, where $K_w = 10^{-14}\ M^2$.

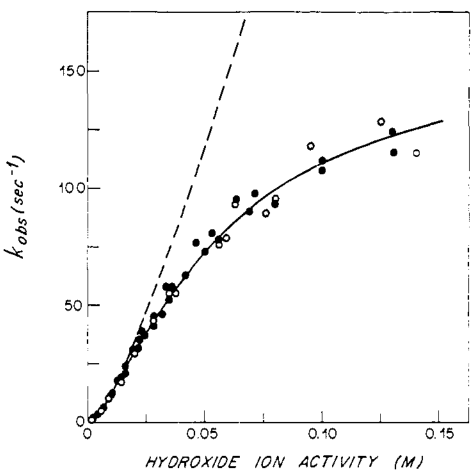


Figure 4. Pseudo-first-order rate constants for the slower phase of DPIC hydrolysis as a function of hydroxide ion activity. The reaction was followed at 320 mμ (open circles) and 255 mμ (filled circles). The solid line is calculated on the basis of the mechanism of eq 5, using the values of the rate constants given in Table II. The dashed line is calculated assuming that the conjugate base of the intermediate does not accumulate significantly under these conditions using eq 6.

Calculations. The calculations are based on the rate equations (eq 4) using the values of the rate constants given in Table II. The concentrations of DPIC, total intermediate, and AEF are calculated as a function of time at given pH values. At 255 mμ (Figure 2), the absorbance contributed by DPIC and AEF is determined from extinction coefficients of $2.5 \times 10^3\ M^{-1}\ \text{cm}^{-1}$ and $5.1 \times 10^3\ M^{-1}\ \text{cm}^{-1}$, respectively, and their calculated concentrations. The difference between the calculated absorbance of DPIC and AEF and the observed absorbance is attributed to the intermediate, and apparent extinction coefficients were determined from the differences in absorbance and the calculated concentrations of the intermediate.

The apparent extinction coefficients of the intermediate were found to be generally constant throughout reactions at any given pH. Values used for the apparent extinction coefficients for the calculated points in Figure 2 are 2.3×10^4, 2.2×10^4, and $1.9 \times 10^4\ M^{-1}\ \text{cm}^{-1}$ for curves 1–3, respectively. The tendency for the apparent extinction coefficients of the intermediate at 255 mμ to increase with increasing pH is consistent with the accumulation of both the neutral intermediate and its conjugate base, assuming that the latter has a higher extinction coefficient in the vicinity of 255 mμ. The possibility that these differences are due to errors in experimental technique and in the calculations has not been ruled out.

For the reactions at 320 mμ (Figure 3), the absorbance of the intermediate and AEF are negligible under the conditions of these experiments. The calculated absorbance values were determined from the calculated concentrations of DPIC and its extinction coefficient of $1.8 \times 10^4\ M^{-1}\ \text{cm}^{-1}$ at 320 mμ.

Calculated pseudo-first-order rate constants for the slower phase of the reactions were obtained from the calculated concentrations of the three species. Identical values of these rate constants are obtained from (1) the slower phase of DPIC disappearance, (2) the decomposition of the intermediate, or (3) the formation of AEF after the initial lag period. The solid line in Figure 4 is from the calculated rate constants according to the mechanism of eq 5.

The accuracy of the values listed in Table II for k_2, K_a, and the ratio k_1/k_{-1} are estimated to be in the range of ±10–15% while the values of k_1 and k_{-1} are probably only accurate to within ±30%.

Ultraviolet Spectrum of the Intermediate. The reaction was followed at different wavelengths ranging from 220 to 340 mμ, at three pH values. Concentrations of DPIC, intermediate, and AEF were calculated from the rate equations as previously described. The ultraviolet spectrum was determined from absorbance measurements at t = 8, 6, and 4 msec at pH 12.00, 12.38, and 12.85, respectively. At the times chosen the initial rapid reactions were essentially completed and errors in the estimation of the age of the solution have relatively small effects on the absorbance attributed to the intermediate. For example, even for the most rapid of the three reactions at pH 12.85, errors of ±1 msec in the estimated age of the solution would cause less than a ±3% variation in the calculated extinction coefficient at 255 mμ. The concentrations of DPIC, total intermediate, and AEF were calculated from the rate equations, and the absorbance of the intermediate was

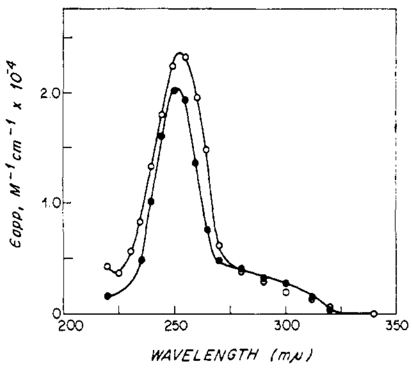


Figure 5. Ultraviolet difference spectrum of the tetrahedral intermediate formed during DPIC hydrolysis. The spectra were determined at pH 12.85 (open circles) and pH 12.00 (filled circles). Molar extinction coefficients were calculated as described in the text.

obtained by subtracting the calculated absorbance of DPIC and AEF from the observed absorbance. The resulting spectra are shown in Figure 5 at pH 12.00 and 12.85. The spectrum at pH 12.38 is similar with extinction coefficients generally intermediate between those at the other two pH values. Each measurement is thought to be accurate within ±0.1 absorbance unit.

The absorbance maximum of the intermediate in this pH range is at 252 ± 2 mμ with a shoulder in the region of 290–300 mμ. Extinction coefficients below 270 mμ appear to increase with increasing pH. This was noted above with the kinetic data at 255 mμ (Figure 2) and may either be attributed to the accumulation of both species of the tetrahedral intermediate or to experimental error. Ultraviolet absorbance data for the intermediate and for two compounds which may be expected to have similar ultraviolet spectra are given in Table III. The extinction coefficient of the intermediate at 252 mμ given in the table is the average of the three determinations. If the differences between the spectra are assumed to be due to conversion of the intermediate to its conjugate base, the spectra of the two species appear to be similar but the extinction coefficients at 252 mμ of the neutral intermediate and its conjugate base would be approximately 1.9 × 10^4 and 2.6 × 10^4 M^{-1} cm^{-1}, respectively.

Table III. Ultraviolet Absorbance of the Tetrahedral Intermediate Formed During DPIC Hydrolysis and Two Related Compounds in Aqueous Solution

	λ_{max} (mμ)	ϵ_{max} (M^{-1} cm^{-1})	ϵ_{max}/ ϵ_{290} mμ
N,N-Dimethylaniline	244	0.90 × 10^4 [a]	8.0
N,N′-Diphenyl-2-carboxyimidazolidine[b]	247	2.1 × 10^4	7.5
N,N′-Diphenyl-2-hydroxyimidazolidine	252 ± 2	~2.2 × 10^4 [c]	~8.0

[a] The extinction coefficient of dimethylaniline should be doubled for comparison with the other compounds. [b] The adduct of N,N′-diphenylethylenediamine with glyoxal at pH 5.20 in 0.05 M sodium acetate buffer, ionic strength 1.0 M with added KCl (R. G. Kallen, personal communication). [c] Average of three values (see text).

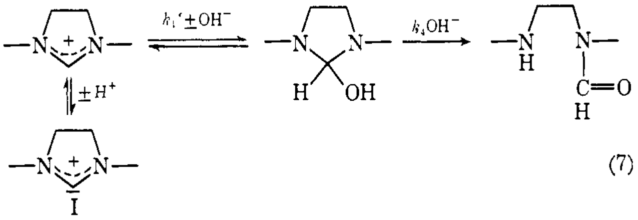
Discussion

The results clearly demonstrate the accumulation of an intermediate during the course of hydrolysis of DPIC. The accumulation of the intermediate increases with increasing pH as shown by absorbance measurements at 255 mμ, near the absorption maximum of the intermediate. The rapid initial loss of absorbance at 320 mμ, where only DPIC absorbs significantly, corresponds to the formation of the intermediate and the large extent of the initial loss of absorbance at high pH values indicates that a large fraction of the initial concentration DPIC is converted to intermediate under these conditions.

The ultraviolet spectrum of the intermediate provides evidence that the intermediate is the tetrahedral addition compound (eq 5). Although the spectrum is not highly accurate, there is clearly a strong peak around 250 mμ and a weak shoulder in the 290–300-mμ range. The location of these bands and their extinction coefficients are similar to 2 mol equiv of dimethylaniline and to the 2-carboxyimidazolidine derivative shown in Table III, compounds which appear to be reasonable models for the tetrahedral intermediate.

Consideration of the Possibility of an Ylide Intermediate. A possible alternative explanation of the results which is considered here is the ionization of the C-2 proton of DPIC with accumulation of the ylide (I) on a side pathway. Although this possibility cannot be completely excluded, it appears unlikely on the basis of the arguments presented below.

1. A scheme involving the accumulation of the ylid intermediate is shown in eq 7. The reactive pathway proceeds through a tetrahedral intermediate under steady state conditions with the k_4 step rate determining,



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and the ylide accumulates on a side pathway during the initial rapid phase of the reaction. According to this scheme the reaction is second order with respect to hydroxide ion activity at low pH but approaches first order at high pH where large amounts of the ylide accumulate. This mechanism cannot account for the observation that the rate constants for the slower phase of the reactions become nearly independent of hydroxide ion activity at high pH. However, it does appear possible to account for the data by this mechanism with a change in rate-determining step from rate-determining breakdown (k_4) to formation (k_1') of the intermediate with increasing pH, in combination with the accumulation of the ylide. It can be seen from inspection of eq 7 that if the k_1' step becomes rate determining at high pH, a large fraction of the starting material would be converted to the ylide during the rapid phase of the reaction and the rates of the slower phases of the reaction would become nearly pH independent. Therefore, the kinetics do not exclude the possibility of accumulation of the ylide intermediate, although it is necessary to invoke a change in rate-determining step as well.

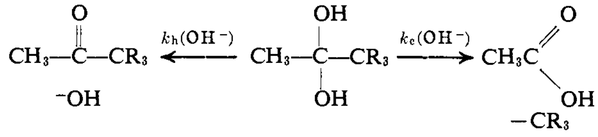
2. The ultraviolet difference spectrum, shown in Figure 5, provides evidence for the tetrahedral intermediate, and it appears unlikely that the spectrum can

be accounted for by the conjugate base of DPIC. The ultraviolet spectrum of the ylide would be expected to resemble that of DPIC, since the conjugated systems in the two structures are similar. The contribution made by the carbene structure (III) to the ultraviolet spectrum of this compound is not known, but we consider it unlikely that the carbene contribution would result in an ultraviolet spectrum similar to that of the observed intermediate.[14]

3. In order to account for the results by a mechanism involving the accumulation of the ylide, the pK_a value for this ionization of approximately 12.3 is required. The pK_a value of this ionization is not known, but the following considerations indicate that a value in the range of 12.3 is unreasonably low.

The hydroxide-catalyzed C-2 proton exchange rate of DPIC has been found to be nearly the same as that of thiazolium salts.[18] This gives an upper limit of approximately 20 for the pK_a of DPIC assuming that the reverse reaction, the protonation of the ylid by water, is diffusion controlled. An approximate pK_a of 20 was estimated by Breslow for thiazolium ions from proton exchange rates, and it was pointed out that this value is an upper limit since the protonation of the thiazolium ylide by water may occur at less than diffusion-controlled rates.[19] Recently, Haake and coworkers have estimated a pK_a value as low as 14 for thiazolium ions, based on the assumption that the rate of protonation of the acetone anion by water ($\sim 10^5$ sec^{-1}) is the same as the rate of protonation of thiazolium ylids by water.[17] Whether this reaction is an appropriate model for the reaction of the thiazolium ylide with water remains open to question. It is well known that carbanions which undergo extensive electronic rearrangement on protonation, *e.g.*, ketones and nitroalkanes, are often protonated at relatively slow rates.

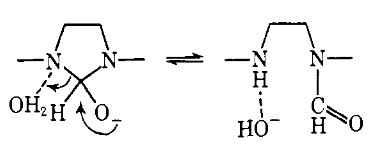
Another estimate of the basicity of thiazolium ions comes from the rates of hydrolysis of diethyl acetylmalonate and 2-acetyl-3,4-dimethylthiazolium ion.[20] The tetrahedral hydrates of these compounds break down to expel hydroxide ion (k_h) or carbanion (k_c) by hydroxide-catalyzed pathways (eq 9). As Lienhard



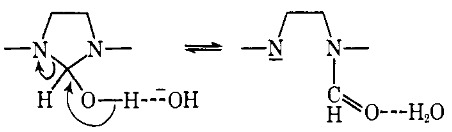
$$\underset{^{-}\mathrm{OH}}{\mathrm{CH_3{-}\overset{O}{\overset{\|}{C}}{-}CR_3}} \xleftarrow{k_h(\mathrm{OH}^-)} \mathrm{CH_3{-}\overset{OH}{\underset{OH}{C}}{-}CR_3} \xrightarrow{k_c(\mathrm{OH}^-)} \underset{-\mathrm{CR_3}}{\mathrm{CH_3C({=}O)OH}} \qquad (9)$$

pointed out, the ratio k_h/k_c for the hydrate of diethyl acetylmalonate is 1.4×10^{-3}. Therefore, the diethyl malonate carbanion (pK_a = 15.2) is expelled $\sim 10^3$ times more rapidly than hydroxide ion (pK_a = 15.7), associated with the slightly lower basicity of this carbanion than hydroxide ion. In contrast, the ratio k_h/k_c for the hydrate of the thiazolium compound is ~ 10. This suggests that the basicity of the thiazolium ylide is greater than that of the diethyl malonate anion by a factor of $\sim 10^4$, giving a pK_a value of ~ 20 for the thiazolium ion.[20] On the basis of the similarity of the C-2 proton exchange in thiazolium salts and in DPIC,[18] the pK_a value of DPIC may also be estimated to be ~ 20. While this can only be considered to be a rough estimate, the indications are that the pK_a of DPIC is well above the 12–13 range. Therefore, it is unlikely that the DPIC ylid accumulates in detectable concentrations under the conditions of the experiments reported here.

Mechanism of the Reaction. The mechanism of eq 5, which accounts for the results of these experiments, was previously proposed for the hydrolysis of DPIC according to the k_3' term in the rate law of eq 2 at pH values below 11, where steady state conditions apply.[4a] The reaction is subject to general base catalysis under these conditions. The k_3' term for hydroxide ion falls close to the line in the Brønsted plot based on catalysis by primary amines, indicating that hydroxide ion acts as a general base (or its kinetic equivalent). It was concluded that the preferred mechanism for the k_3' reactions is the general acid catalyzed breakdown of the conjugate base of the tetrahedral intermediate. In the case of hydroxide ion the reaction is catalyzed by water as shown in mechanism IV.[21] The alternative mechanism (V) involving proton removal from the neutral intermediate by a general base was previously ruled out because the aniline anion which is the initial product of this reaction is too unstable to occur on the reaction pathway as a free intermediate.



IV



V

The present results provide further evidence for these conclusions. In order to account for the reaction by mechanism V, the fact that the rate constants for the

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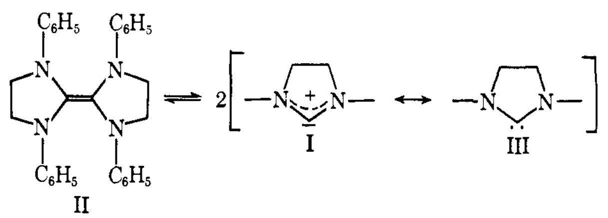
[14] Evidence has been presented by Wanzlich that the conjugate base of DPIC is formed by the dissociation of the compound, bis(1,3-diphenyl-2-imidazolidinylidene) (II), to the monomer ylide–carbene (I ↔ III) at elevated temperatures (eq 8).[15] Several reactions of II were postulated



(8)

to occur through the monomer, although it has also been pointed out that a direct attack on the dimer (II), without a monomer intermediate, has not been ruled out in some cases.[16] The reactivity of II indicates that the monomer is predominantly a nucleophilic agent and does not undergo certain reactions typical of carbenes. It cannot be concluded from these observations that the carbene structure makes a major contribution to the structure of the conjugate base of DPIC, and the apparent nucleophilic character of the latter suggests that the ylid form is important. Although the conjugate base of DPIC has been referred to as a stable carbene,[16] its relative stability is presumably related to a large contribution of the ylid form to its structure.[17]

[15] H. W. Wanzlick, *Angew. Chem. Intern. Ed. Engl.*, **1**, 75 (1962).

[16] J. Hine, "Divalent Carbon," The Ronald Press Co., New York, N. Y., 1964, pp 165–168.

[17] P. Haake, L. P. Bausher, and W. B. Miller, *J. Amer. Chem. Soc.*, **91**, 1113 (1969).

[18] W. Haffert, R. Lundin, and L. Ingraham, *Biochemistry*, **2**, 1298 (1963).

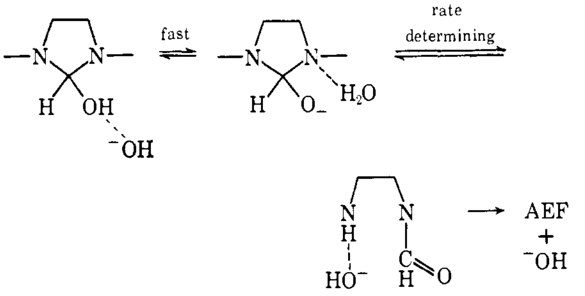
[19] R. Breslow, *Ann. N. Y. Acad. Sci.*, **98**, 445 (1962).

[20] G. E. Lienhard, *J. Amer. Chem. Soc.*, **88**, 5642 (1966).

[21] The value of k_3' (B = OH$^-$) calculated from the relation, $k_3' = k_1k_2K_a/k_{-1}K_w$, is 12.5×10^6 M^{-2} min^{-1}. The difference between this value and the previously reported value of 9.0×10^6 M^{-2} min^{-1} is attributed to experimental errors, particularly in the earlier work.[4a]

slower phase of the reaction become nearly pH independent at high pH values (Figure 4), requires the anionic conjugate base of the intermediate to be unreactive. This leads to the unreasonable conclusion that the expulsion of the leaving group aided by partial removal of the hydroxyl proton in the transition state (V) is a more favorable reaction than the expulsion of the leaving group from the ionized intermediate (IV), even with the obvious advantage of general acid catalysis of the expulsion of the leaving group in mechanism IV. At pH values well below the pK_a of the intermediate, where only a small fraction of the total intermediate is present in the anionic form, even a favorable reaction of this species may be undetectable. At pH values in the vicinity of the pK_a of the intermediate the reactivity of the anionic intermediate would have to be substantially less than that of the neutral intermediate to escape detection.

Another mechanism which was also previously considered for this reaction under steady state conditions was the one-encounter mechanism shown in eq 10. In this mechanism, a single catalyst molecule both removes and donates a proton during the timing of a single encounter with the substrate, avoiding the formation of the anionic species as a free intermediate. Similar mechanisms have been proposed for the hydrolysis of acetals[22] and for the addition of hydroxylamine to aldehydes.[23] It was shown that stepwise proton transfers could not account for the observed rates of these reactions. One-encounter mechanisms must be considered in other carbonyl and acyl group reactions, even if mechanisms involving stepwise proton transfer cannot be ruled out. These mechanisms should be distinguished from bifunctional catalysis, in which two protons are transferred in the same transition state.[24]



(10)

The objections to the one-encounter mechanism for DPIC hydrolysis (eq 10) are similar to those raised for mechanism V. The free anionic intermediate should react at least as readily by mechanism IV as the neutral species reacts by the one-encounter mechanism. Since the one-encounter mechanism involves the neutral intermediate it would be necessary to postulate that the contribution of the anionic intermediate to the reaction rate is negligible at pH values where over half of the intermediate is in the anionic form, in order to account for the pH dependence of the slower phase of reaction.

A Possible Diffusion-Controlled Rate-Determining Step. It was previously reported that the k_3' term for the catalysis of DPIC hydrolysis by water ($v = k_3'$·[DPIC][OH$^-$][H$_2$O], eq 2) deviates below the value predicted by the Brönsted plot of the k_3' terms for other bases by a factor of at least 30. The actual deviation is larger than this because the k_3' term for water is ambiguous and includes a contribution from the k_2' term for hydroxide ion ($v = k_2'$[DPIC][OH$^-$]). Based on the mechanism of eq 5, the rate-determining step of the reaction according to the k_3' term for water is the proton-catalyzed decomposition of the anionic tetrahedral intermediate to form the product. We shall refer to this reaction by the rate constant k_{2,H^+} to distinguish it from the rate constant for the uncatalyzed or water-catalyzed reaction, k_2 in eq 5. It was previously suggested, on the basis of assumed values for k_1/k_{-1} and K_a in eq 5, that the negative deviation of the k_3' term for water could be explained by a diffusion-controlled reaction.[4a] We can now calculate k_{2,H^+} from eq 11 and the values of the constants given in Table II, using the previously reported value of 0.15 M^{-2} sec^{-1} for k_3'.[25] The calculated value of k_{2,H^+} is

$$k_{2,\mathrm{H}^+} = k_3'k_{-1}[\mathrm{H_2O}]/k_1K_a \qquad (11)$$

7×10^{11} M^{-1} sec^{-1}, which is approximately two orders of magnitude greater than a diffusion-controlled rate. This is consistent with the interpretation that the k_3' reaction is diffusion controlled and that most of the observed rate is accounted for by the kinetically equivalent k_2' term for hydroxide ion. Similar calculations indicate that catalysts with pK values less than three will react at diffusion-controlled rates. If the buffer catalysis is interpreted as general acid catalysis of the k_2 step, the Bronsted plot for the observed reactions has a slope (α value) of 0.74 for catalysts with pK values above 4. The calculations indicate that there is a break in the plot at pK of 3 where the k_2 step becomes diffusion controlled and stronger acids would follow a Brønsted slope of zero. The catalysts previously reported all have pK_a values above 4 with the exception of the proton, but the proton-catalyzed reaction indicates that a break in the plot must occur.

This behavior is similar to that seen with the base-catalyzed decomposition of hemithioacetals.[26] Brønsted slopes are 0.80 for these reactions, for catalysis by bases with pK values below 7. The hydroxide-catalyzed reactions deviate below these plots and occur with rate constants of approximately 10^{10} M^{-1} sec^{-1} and values of $\Delta H^{\ddagger}$ of 2 kcal/mol. Simple proton transfer reactions have been shown by Eigen to have Brønsted slopes of 1.0 when the rates are less than diffusion controlled and to level off to a slope of zero at the diffusion-controlled limit.[27] The fact that slopes of Brønsted plots for hemithioacetal decomposition and for DPIC hydrolysis are less than 1.0 (0.80 and 0.76, respectively) indicates that proton transfer alone is not rate determining for these reactions. These are examples of carbonyl and acyl group reactions occurring with rate-determining, diffusion-controlled encounter, but the overall reaction rates are relatively slow because the rate-determining steps are preceded by unfavorable equilibria. It has been recently shown that the S to N acetyl transfer of S-acetylmercaptoethylamine occurs with rate-deter-

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(22) M. Eigen, *Discussions Faraday Soc.*, 39, 7 (1965).

(23) J. E. Reimann and W. P. Jencks, *J. Amer. Chem. Soc.*, 88, 3973 (1966).

(24) For example, see B. A. Cunningham and G. L. Schmir, *ibid.*, 88, 551 (1966).

(25) This value was previously reported as the k_2' term for hydroxide ion (500 M^{-1} min^{-1}).[4a]

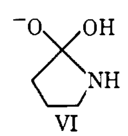
(26) R. Barnett and W. P. Jencks, *J. Amer. Chem. Soc.*, 89, 5963 (1967).

(27) M. Eigen, *Angew. Chem. Intern. Ed. Engl.*, 3, 1 (1964).

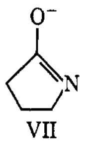
mining diffusion-controlled proton transfer although the overall rates are also relatively slow.[28]

Tetrahedral Intermediates Derived from Acyl Compounds. There is a large body of evidence from kinetic and exchange experiments that several (but by no means necessarily all) acyl transfer reactions proceed through tetrahedral addition intermediates.[2] Furthermore, there are many known tetrahedral addition compounds at the acyl level of oxidation which are stable. The latter include ortho esters, orthoamides, and related compounds,[29] the adduct of ethoxide with ethyl trifluoroacetate,[30] and certain cyclic tetrahedral compounds.[31] Nevertheless, it appears that the direct observation of a tetrahedral intermediate during the course of an acyl transfer or hydrolysis reaction has not been clearly demonstrated previously. Addition compounds of aldehydes and ketones during nucleophilic reactions have been frequently observed, and this difference between carbonyl and acyl group reactions can be, at least in part, attributed to greater resonance stabilization of acyl compounds.[2]

In this connection, Vinnik and Moiseyev have concluded that a tetrahedral intermediate (VI) is observed spectrophotometrically during the hydrolysis of γ-butyrolactam in concentrated aqueous potassium hydroxide solutions.[32] The carbonyl stretching frequency (amide I band) of γ-butyrolactam at 1665 cm^{-1} (H_2O) was reported to disappear in >10–15% potassium hydroxide and a new band of comparable intensity is observed at 1555 cm^{-1}. A preparation of the sodium salt of γ-butyrolactam (VII) was shown to have a strong absorption band at 1670 cm^{-1} and a weak band at 1555 cm^{-1} both in a KBr pellet and in absolute alcohol solution. These investigators conclude that these results rule out the possibility that the observed intermediate is the conjugate base of γ-butyrolactam (VII). We find this interpretation unconvincing. The similarity of the infrared spectra reported for γ-butyrolactam and its sodium salt suggests that the sodium salt may have become protonated under the conditions of these experiments. Furthermore, there appears to be little reason to expect that the pseudobase of γ-butyrolactam (VI) would possess an absorption band in the vicinity of 1555 cm^{-1} with an intensity comparable to that of the amide I band γ-butyrolactam.

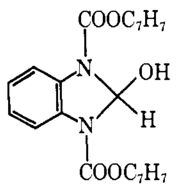


VI

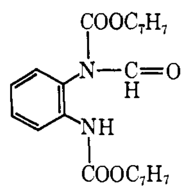


VII

The isolation of the imidazolinium pseudobase, 1,3-dicarbobenzoxy-2-hydroxybenzimidazoline (VIII), from a water-benzene mixture at 0° has been reported.[33] An elemental analysis and a strong absorption band at 3.0 μ ($CHCl_3$), attributed to the hydroxyl group, are cited as evidence for this structure. It appears possible that this compound is the imide (IX), analogous to AEF. Even if the pseudobase structure is the correct one, its stability in water is unclear.



VIII



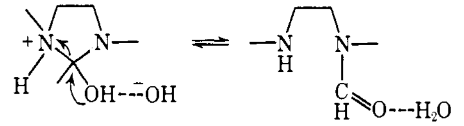
IX

Stability of the Tetrahedral Intermediate and Related Reactions. The apparent standard free energy of hydrolysis of the intermediate at pH 7.0, which provides a measure of its stability under physiological conditions, is calculated as indicated in Table IV. The free energy of formation of the intermediate from DPIC of +7.1 kcal/mol is based on the values of k_1 and k_{-1} given in Table I. This value, in combination with the free energy of hydrolysis of DPIC to form AEF, gives +9.8 kcal/mol for the free energy of formation of the intermediate from the anilide, AEF. Assuming a value of the free energy of hydrolysis of the amide group of AEF of *ca.* −2 kcal/mol, gives a value for the standard free energy of hydrolysis of the intermediate of approximately −12 kcal/mol at pH 7.0. The anionic form of the intermediate is less stable than the neutral species by some 7.9 kcal/mol at pH 7.0. This energy barrier is apparently avoided by the reaction corresponding to the k_2' term of the rate law (eq 2), according to mechanism X, which involves general base catalyzed removal of the hydroxyl proton from the protonated intermediate in the rate-determining step.[4a]

Table IV. Apparent Standard Free Energy Changes at 25.0° [a]

Reaction	K^b	$\Delta F_{\mathrm{pH}}{}^{\circ\prime\,b,c}$ 7.0, kcal/mol
1. DPIC + $H_2O \rightleftharpoons$ TH + H^+	6.4×10^{-13} *M*	+7.1
2. DPIC + $H_2O \rightleftharpoons$ AEF + H^+	1.1×10^{-5} *M*	−2.7
3. AEF $\rightleftharpoons$ TH	5.8×10^{-8}	+9.8

[a] Ionic strength maintained at 1.0 *M* with added KCl. [b] Activity of water is assumed to be equal to 1.0. [c] Standard states are 1.0 *M* with the exception of H^+ which is 10^{-7} *M*.



X

Tetrahedral intermediates have been detected for the aminolysis of amides[34] and imidoesters[35] from kinetic experiments and exchange reactions. These intermediates are similar to the intermediate described here for DPIC hydrolysis and their formation is presumably subject to similar mechanisms of general acid–base catalysis.

The experiments reported here may aid in the understanding of certain enzyme-catalyzed reactions. The hydrolysis of N^5,N^{10}-methenyltetrahydrofolate to N^{10}-formyltetrahydrofolate catalyzed by the enzyme cyclo-

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(28) R. E. Barnett and W. P. Jencks, *J. Amer. Chem. Soc.*, **91**, 2358 (1969).

(29) See ref 25 and 26 in ref 4a.

(30) M. L. Bender, *J. Amer. Chem. Soc.*, **75**, 5986 (1953).

(31) H. E. Zaugg, V. Papendick, and R. J. Michaels, *J. Amer. Chem. Soc.*, **86**, 1399 (1964).

(32) See M. I. Vinnik and Y. V. Moiseyev, ref 3.

(33) A. Patchornik, A. Berger, and E. Katchalski, *ibid.*, **79**, 6416 (1957).

(34) W. P. Jencks and M. Gilchrist, *ibid.*, **86**, 5616 (1964).

(35) E. S. Hand and W. P. Jencks, *ibid.*, **84**, 3505 (1962).

hydrolase is particularly pertinent. Several aminohydrolases are known which catalyze the hydrolysis of cyclic amidine groups to amides in cytosine, purines, and their derivatives. Two enzyme reactions which involve amide aminolysis are the transfer of formyl groups from N^{10}-formyltetrahydrofolate to phosphoribosylaminoimidazolecarboxamide, on the pathway of purine biosynthesis, and from the same coenzyme to methionyl-tRNA, for the initiation protein synthesis in bacteria. There is apparently little information available on the mechanisms of these enzyme reactions at the present time. It is of interest that in recent work by Caplow the substituent effects on the hydrolysis of esters and anilides by chymotrypsin have been interpreted as evidence for the existence of tetrahedral intermediates during the enzymatic hydrolysis of these compounds.[36]

Acknowledgments. The author is grateful to Dr. W. P. Jencks for helpful discussions and for the use of a stopped-flow apparatus. The ultraviolet spectrum of N,N′-diphenyl-2-carboxyimidazolidine was kindly provided by Dr. R. G. Kallen. Competent technical assistance was given by Miss Libby Servello.

(36) M. Caplow, *ibid.*, **91**, 3639 (1969).

Nuclear Magnetic Resonance Studies of the Interaction of *trans*-Cinnamate with α-Chymotrypsin

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Abstract: A high-resolution proton magnetic resonance study of the Michaelis complex formed between cinnamate ion and the proteolytic enzyme, α-chymotrypsin, has been carried out. It has found that the aromatic proton resonances of the complexed acid anion are shifted upfield 0.6–0.8 ppm while the vinyl protons are changed very little. Line-broadening effects are consistent with the suggestion that this acid is bound tightly enough to the enzyme that it does not have any freedom of motion independent of the motion of the enzyme. Experiments with enzymes which have been chemically modified at the active site indicate that this site is also the locus of the cinnamic acid–enzyme interaction.

The mechanism of the α-chymotrypsin-catalyzed hydrolysis of esters and amides can be represented by eq 1.[2] Although likely an oversimplification, this representation is intended to portray, as the initial phase of the reaction, a reversible complexation of the substrate, S, with the enzyme, E, to give an enzyme–substrate complex, ES, within which the acyl group of the substrate is transferred to the protein and the alcohol or amino product (P_1) is released. The resulting acylated enzyme, ES′, undergoes hydrolysis to form the product acid (P_2) and to regenerate the free enzyme. An additional equilibrium involving a complex between the enzyme and the acid product (EP_2) may intervene along the reaction paths leading from the acylated enzyme to products.

$$E + S \rightleftharpoons ES \rightleftharpoons \underset{\substack{+\\ P_1}}{ES'} \rightleftharpoons EP_2 \rightleftharpoons E + P_2 \qquad (1)$$

A reliable understanding of the physico-chemical interactions which are responsible for the specificity with which substrates and products are attracted to this enzyme is not yet available although a considerable amount of data has been accumulated. It now appears that magnetic resonance spectroscopy will play an important role in clarifying the nature of these interactions[3-6] since the parameters which characterize a magnetic resonance spectrum can reflect not only the nature of a spin's environment but also the time stability of this environment.

A number of elegant experiments by Bender and coworkers have shown that, when the acyl group is cinnamoyl, the corresponding acylated enzyme (ES′) is stable under a rather wide range of conditions.[7] Moreover, *trans*-cinnamic acid (I) is a good competitive inhibitor of the enzyme and thus participates in an enzyme–product equilibrium of the type shown in eq 1.[8,9] The cinnamoyl-α-chymotrypsin system is fairly well understood from the viewpoints of traditional kinetic and ultraviolet spectroscopic experiments and is, therefore, a logical choice for further investigation by magnetic resonance techniques. We describe here the results of a high-resolution nmr study of the interaction of the anion of cinnamic acid with bovine α-chymotrypsin.

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(1) On leave from the College of Wooster, Wooster, Ohio, 1968–1969.

(2) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," W. A. Benjamin, Inc., New York, N. Y., 1966, p 213.

(3) T. M. Spotswood, J. M. Evans, and J. H. Richards, *J. Amer. Chem. Soc.*, **89**, 5052 (1967).

(4) E. Zeffren and R. E. Reavill, *Biochem. Biophys. Res. Commun.*, **32**, 73 (1968).

(5) B. D. Sykes, *J. Amer. Chem. Soc.*, **91**, 949 (1969).

(6) L. J. Berliner and H. M. McConnell, *Proc. Natl. Acad. Sci. U. S.*, **55**, 708 (1966).

(7) M. L. Bender and B. Zerner, *J. Amer. Chem. Soc.*, **84**, 2550 (1962).

(8) J. Bongal and F. Calvert, *Rev. Espan. Fisiol., Suppl.*, **2**, 177 (1960); *Chem. Abstr.*, **55**, 4754 (1961).

(9) M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Amer. Chem. Soc.*, **84**, 2540 (1962).