Kinetics and Mechanism of the Bamberger Cleavage of Imidazole and of Histidine Derivatives by Diethyl Pyrocarbonate in Aqueous Solution

M. E. Grace, M. J. Loosemore, M. L. Semmel, and R. F. Pratt*

Contribution from the Department of Chemistry, Wesleyan University, Middletown, Connecticut 06457. *Received December 26, 1979*

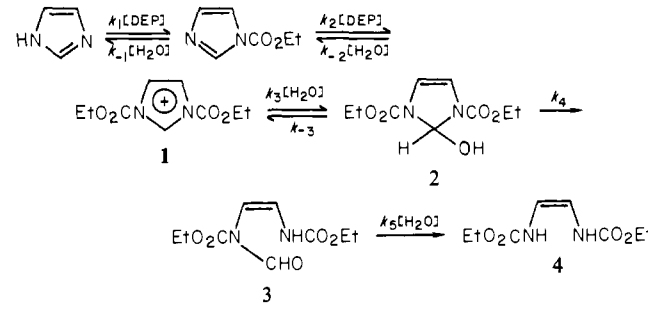
Abstract: The kinetics and mechanism of the Bamberger cleavage of imidazole and certain histidine derivatives by diethyl pyrocarbonate in dilute aqueous solution have been studied. The cleavage yields have been measured as a function of reagent concentrations and of pH, and the cleavage rates have been measured as a function of reagent concentrations. At low diethyl pyrocarbonate concentrations (<10 mM) in phosphate buffer at pH 6.0 carbethoxylation of *N*-carbethoxyimidazole is rate determining to the cleavage while at higher diethyl pyrocarbonate concentrations breakdown of an intermediate becomes rate determining. The intermediate has been shown to be 2-hydroxy-1,3-dicarbethoxy-4-imidazoline, an unusually stable ($t_{1/2} \simeq$ 2 min at pH 6.0) hydroxyl-substituted carboxylic acid derivative tetrahedral adduct. Other compounds of this type have been prepared and characterized.

Diethyl pyrocarbonate (diethyl dicarbonate, ethoxyformic anhydride, DEP) is probably the most commonly used reagent for the specific modification of histidine residues in proteins.[1,2] The product of the modification is normally considered to be a *N*-carbethoxyhistidine derivative whose absorption at 240 nm allows quantitation of the histidine modification that has occurred.[3,4] We[5] and others[6,7] however have shown that it is also possible to achieve the Bamberger cleavage of imidazole rings under the mild conditions of protein modification. The presence of this cleavage reaction, on one hand, can interfere with the estimation of the number of histidine residues modified by DEP since the products of the cleavage also absorb at 240 nm[6,7] but, on the other, opens up the possibility of a specific, irreversible histidine modification which would be more useful than reversible modification in a number of applications. In the present paper we describe the results of experiments designed to explore further the conditions under which the cleavage occurs and to examine its kinetics and mechanism in some detail with a view toward the design of further, more specific reagents. Most of these experiments pertain to the model compound imidazole, but some results obtained with histidine derivatives are also described.

Results and Discussion

The Bamberger cleavage of imidazole by DEP showing products and proposed intermediates[5,7] is shown in Scheme I. The overall reaction has been shown to proceed under conditions of excess DEP and neutral pH and at ambient temperatures.[5-7] Under these conditions the products *N,N'*-dicarbethoxy-*N*-formyl-1,2-diaminoethene (**3**) and *N,N'*-dicarbethoxy-1,2-diaminoethene (**4**) can be isolated.[5] The reaction can either be carried out in homogeneous aqueous solution with imidazole concentrations of the order of those used of proteins in chemical modification experiments (0.01–0.1 mM) or more conveniently on a preparative scale using a two-phase reaction system. Details of the latter along with characterization of the products are presented in the Experimental Section of this paper. In either case the reaction can be made quantitative under conditions described there and below. Preparatively at pH 6 and with short reaction times recovery of **3** is close to quantitative, but at higher pH and with longer reaction times a mixture of **3** and **4** or even **4** alone is obtained. Pure **3** is quantitatively converted into **4** under these same conditions,

Scheme I



slowly ($t_{1/2} \simeq$ 5 h) at pH 6 and more rapidly at higher pH. The products **3** and **4**, as described previously,[5] are characterized by strong absorption maxima at low wavelength and still substantial absorption at 240 nm where the latter is responsible for problems in the use of DEP for histidine modification of proteins.

The effect of reaction conditions on the rate and extent of imidazole cleavage was studied in some detail (Figures 1–3). The rate was measured by the maximal (attained after an induction period; see ref 5 and below) rate of appearance of **3**, as measured by absorbance at 215 nm, whereas the extent of cleavage was measured by the yield of **4**, determined as described in the Experimental Section. Also shown in these figures are some corresponding data for the reaction of DEP with *N*-carbethoxyimidazole under the same conditions which of course yields the same products **3** and **4**. All of these reactions were essentially complete; i.e., no further spectral changes were observed, in 1 h. At pH 6.0 and in phosphate buffer neither the yields nor the rates were significantly affected by changes in phosphate concentration (0.01–0.2 M) or ionic strength (0.1–0.6 M). As indicated by Figure 3 there is little indication that the change in buffer from phosphate to acetate or to pyrophosphate affected the extent of reaction. In 0.1 M tris(hydroxymethyl)aminomethane buffer at pH 7.5 however no cleavage was observed. Presumably aminolysis of DEP (or of *N*-carbethoxyimidazole or **1**) by Tris is faster than the cleavage reaction.

The data of Figures 1–3 indicate firstly that at pH 6.0 in 0.1 M phosphate buffer imidazole cleavage is essentially complete in the presence of 10 mM DEP although not at concentrations significantly below this; the yield is only about 40% at 1 mM for example. With 10 mM DEP there is little variation in yield with pH between pH 5 and pH 9. At a lower DEP concentration however (1 mM) it is clear that the maximal yield is achieved with pH between 6 and 7. In view of the probable complexity of the mechanism (Scheme I) with all of its potential for specific- and general-acid base catalysis and with all steps as far as **2** potentially reversible, a large number of factors could contribute

(1) E. W. Miles, *Methods Enzymol.*, **48**, 431–443 (1977).
(2) F. Schneider, *Angew. Chem., Int. Ed. Engl.*, **17**, 583 (1978).
(3) A. Muhlrád, G. Hegyi, and G. Toth, *Acta Biochim. Biophys. Acad. Sci. Hung.*, **2**, 19 (1967).
(4) J. Ovádi, S. Libor, and P. Elodi, *Acta Biochim. Biophys. Acad. Sci. Hung.*, **2**, 455 (1967).
(5) M. J. Loosemore and R. F. Pratt, *FEBS Lett.*, **72**, 155 (1976).
(6) J. F. G. Vliegenthart and L. Dorland, *Biochem. J.*, **117**, 31P (1970).
(7) S. N. Avaeva and V. I. Krasnova, *Bioorg. Khim.*, **1**, 1600 (1975).

0002-7863/80/1502-6784$01.00/0

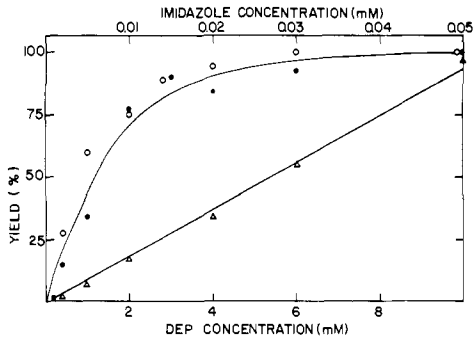


Figure 1. The yield of Bamberger cleavage products at pH 6.0 from the reaction of varying DEP concentrations with 0.02 mM imidazole (●) and *N*-carbethoxyimidazole (○) and of 10 mM DEP with varying concentrations of imidazole (△).

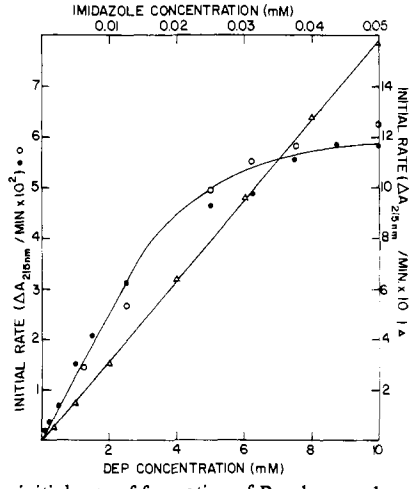


Figure 2. The initial rate of formation of Bamberger cleavage products at pH 6.0 from the reaction of varying DEP concentrations with 0.02 mM imidazole (●) and *N*-carbethoxyimidazole (○) and of 10 mM DEP with varying concentrations of imidazole (△).

to the pH profile of Figure 3. Some of course are obvious: a competing reaction at all pHs must be the hydrolysis of DEP ($t_{1/2} \simeq 20$ min at pH 6 in 0.1 M phosphate[8] and presumably smaller at higher pH) which is presumably successful at both low, where imidazole is protonated, and high pH. At 1 mM DEP considerable amounts of *N*-carbethoxyimidazole ($\lambda_{max} = 232$ nm but labile to Tris) were produced at pHs above 6. Apparently here the DEP concentration was not high enough to drive the cleavage reaction to completion before hydrolysis of the DEP had occurred. This suggests, as would be expected and as discussed further below, that the second step involving DEP is more refractory than the first.

The rate data of Figure 2 suggest that, over the concentration ranges employed (0–10 mM DEP, 0–0.1 mM imidazole) the rate of appearance of **3** is first order in imidazole which is as expected. However the order with respect to DEP changes. At low DEP concentration, the rate appears linear, i.e., first order in DEP, while at high DEP concentration, the rates become independent of DEP concentration. A change in rate-determining step has thus probably occurred, from one first order in DEP to one zero order. The rate-determining step first order in DEP is step 2. Both chemical intuition and the accumulation of *N*-carbethoxyimidazole mentioned above suggest this. Further the same rate behavior is seen (Figure 2) when *N*-carbethoxyimidazole rather than imidazole is used as starting material and hence the rate-determining step must be beyond step 1.

Since at high DEP concentration a step not involving DEP must be rate determining, an intermediate subsequent to *N*-carbethoxyimidazole must accumulate in solution. In agreement with

(8) S. Osterman-Golkar, L. Ehrenberg, and F. Solymosy, *Acta Chem. Scand. B*, **28**, 215 (1974).

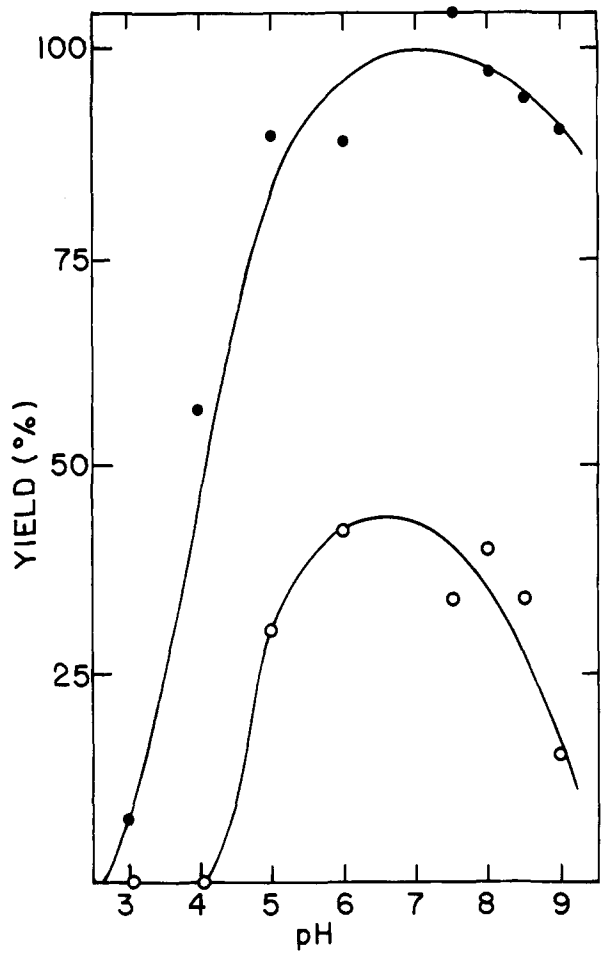


Figure 3. The yield of Bamberger cleavage products with varying pH from reaction of 10 mM (●) and 1 mM (○) DEP with 0.02 mM imidazole. The buffers used were acetate (pH 4–5), phosphate (pH 6–7.5), and pyrophosphate (pH 8–9).

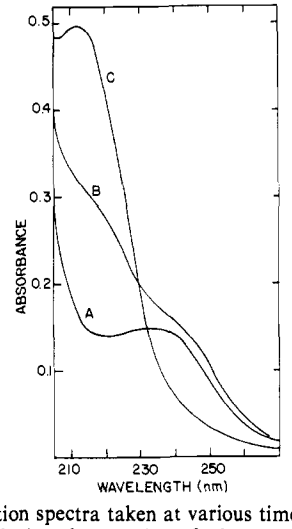


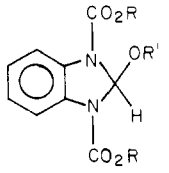
Figure 4. Absorption spectra taken at various times (A, 1 min; B, 3.5 min; C, 20 min) during the reaction of 10 mM DEP with 0.02 mM imidazole at pH 6.0.

this conclusion an induction period was observed in the appearance of **3**, both at low DEP concentration (presumably due to accumulation of *N*-carbethoxyimidazole) and at high DEP concentration. That an intermediate does accumulate at high DEP concentrations is seen directly from a series of spectra taken rapidly after the addition of DEP (10 mM) to imidazole (0.02 mM), one of which is shown in Figure 4. The intermediate, absorbing maximally at around 235 nm ($\epsilon \simeq 7000$, this being the value obtained by assuming complete conversion), forms rapidly ($t_{1/2}$

≃ 25 s at 10 mM DEP) and then decays to form **3**. The existence of a tight isosbestic point at 231 nm between this intermediate and **3** indicates that no further intermediate accumulates along this part of the reaction sequence. The same intermediate is observed when *N*-carbethoxyimidazole is used rather than imidazole.

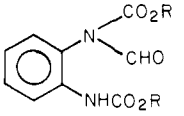
Scheme I identifies the observed intermediate as either **1** or **2**, although the appearance of either of these as a relatively long-lived (minutes) species would be something of a surprise; **1**, a 1,3-diacylimidazolium ion, must certainly have a very high acyl-transfer potential such that in aqueous solution the equilibrium between it and *N*-carbethoxyimidazole would be expected to be well toward the latter and **2** is a carboxylic acid derivative tetrahedral adduct, of a type not commonly observed, despite a flurry of recent sightings.[9]

The absorption spectrum of the intermediate appears quite similar to that of **4**, suggesting that the intermediate might be **2** rather than **1**, although the spectrum of the latter is not easily predictable—1,3-diacylimidazolium ions have not been isolated to our knowledge. A survey of the literature on heterocyclic pseudobases[10] gave no examples of stable hydration between two nitrogen atoms; likewise 2-hydroxy-4-imidazolines seem poorly characterized. There is however the report of Patchornik et al.[11] of the isolation of 1,3-dicarbobenzoxy-2-hydroxybenzimidazoline (**5a**) from the reaction of benzimidazole with benzylchloroformate



5a, R = CH_2Ph, R' = H
5b, R = Et, R' = H
5c, R = Et, R' = Et

in a water–benzene mixture. Since the evidence for the structure given is scant—an elemental analysis and a strong absorption band at 3 μm, attributed to the hydroxyl group—Robinson[12] suggested that this compound could just as easily be the ring-opened imide **6a**, analogous to **3**. We prepared a compound in the manner



6a, R = CH_2Ph
6b, R = Et

described by Patchornik et al.[11] which has the same properties, melting point and infrared absorption, as they report and which could be recrystallized unchanged from hexane/benzene. The NMR spectrum of this compound however did not contain the characteristic downfield formyl proton resonance that would be expected of **6a** (and as seen in **3**) and did contain a single type of benzylic proton resonance. The compound isolated then is most likely the originally suggested **5a**. The methine resonance is not seen in the spectrum and thus must occur under the aromatic multiplet; such a chemical shift is reasonable and the assignment is supported by the integration of the aromatic region.

Treatment of **5a** in acetonitrile with a catalytic amount of triethylamine converts it into an oil with an NMR spectrum as would be expected for **6a**, with a clear formyl resonance at δ 9.2 and two separate benzylic resonances. Since the other properties of this compound were also in accord with structure **6a** there seems little doubt that the compound isolated by Patchornik et al.[11] is the hydroxyimidazoline but that the latter is readily converted with base catalysis into **6a**.

Table I. First-Order Rate Constants for Reactions of Tetrahedral Adducts at pH 6.0

reaction	$10^3 k_{obsd}$, s^{-1}	reaction	$10^3 k_{obsd}$, s^{-1}
2[a] → 3	6.4 ± 0.4	5b → 6b	10.2
2[b] → 3	6.0 ± 0.5	5c → 5b	0.058
7 → 2[c]	1.9 ± 0.2		

[a] Intermediate observed in the imidazole/DEP reaction. [b] Intermediate observed in the *N*-carbethoxyimidazole/DEP reaction. [c] Derived from kinetic analysis–see text.

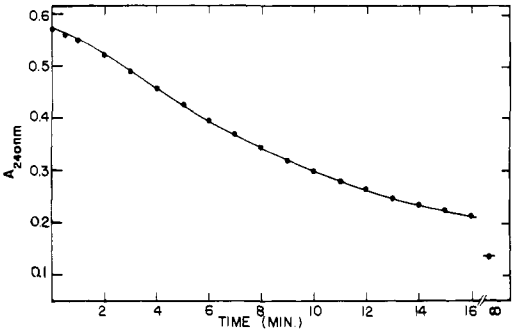
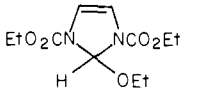


Figure 5. A plot of absorbance at 240 nm as a function of time measured during the hydrolysis of **7** at pH 6.0. The line is experimental, and the points are calculated as described in the text.

Attempts to examine the behavior of **5a** in aqueous solution were thwarted by the insolubility of this compound, so the synthesis of carbethoxy analogue **5b** was attempted. Under the same conditions as employed for the preparation of **5a**, but with use of ethyl rather than benzyl chloroformate, a compound with properties corresponding to **5b** could be isolated. With imidazole however the analogous compound **2** could not be isolated. Under the same conditions as above **3** was the principal product but with the reaction pH held at 6 rather than 8; an appreciable amount of a second product was also formed. It was also found that the yield of the second product could be increased, still at pH 6, by employment of DEP rather than ethyl chloroformate in the preparation. The spectra and analyses reported (Experimental Section) identify this product as **7**. Pertinent to the identification



7

are the UV spectrum with λ_{max} = 240 nm and ε = 6600 (cf. the spectrum of **4** and that of Figure 4), the NMR spectrum with two types of ethyl groups in a 2:1 ratio, with two equivalent olefinic protons, and with a low field (δ 6.60), one proton singlet attributable to the methine proton, and the mass spectrum with the parent ion at *m/e* 258 and a strong peak at *m/e* 140 corresponding to elimination of diethyl carbonate to form the stable 1-carbethoxyimidazole ion. Analogously **5c** could be prepared from benzimidazole and DEP. The formation of ethoxy derivatives **5c** and **7** under these conditions can be understood from the observation of Patchornik et al.[11] that **5a** could be converted into the analogous methoxy derivative by its reaction with methanol in benzene; in the present case ethanol is supplied by hydrolysis of ethyl chloroformate and DEP or by reaction of the latter with imidazole or benzimidazole.

In 0.1 M phosphate buffer at pH 6.0, **5b** and **5c** were both converted in first-order processes with rate constants given in Table I into a compound whose absorption spectrum identifies it as **6b**. The kinetics of the hydrolysis of **7** proved slightly more complex. Under the above conditions the absorbance at 240 nm decreased with time while that at lower wavelength increased. The product of the reaction with a λ_{max} of 215 nm was clearly **3**. However the decrease in absorbance at 240 nm was not first order, as shown

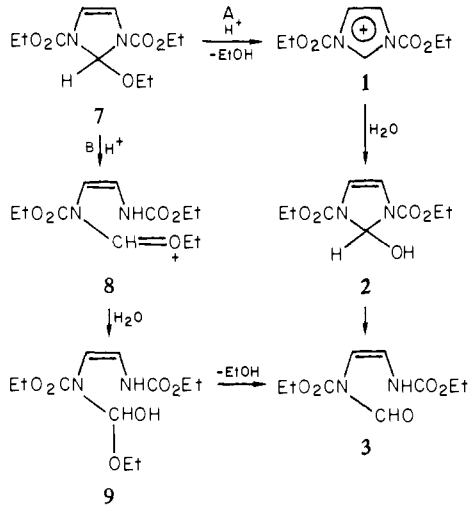
(9) M. Ahmad, R. G. Bergstrom, M. J. Cashen, Y. Chiang, A. J. Kresge, R. A. McClelland, and M. F. Powell, *J. Am. Chem. Soc.*, **101**, 2669 (1979); F. Khouri and M. K. Kaloustian, *Ibid.*, **101**, 2248 (1979), and references therein.

(10) A. Albert and W. L. F. Armarego, *Adv. Heterocycl. Chem.*, **4**, 1 (1965); A. Albert, *Ibid.*, **20**, 117 (1976).

(11) A. Patchornik, A. Berger, and E. Katchalski, *J. Am. Chem. Soc.*, 79, 6416 (1957).

(12) D. R. Robinson, *J. Am. Chem. Soc.*, **92**, 3138 (1970).

Scheme II



in Figure 5. An induction period in the formation of **3** was also observed. This indicates a two-step reaction with accumulation of an intermediate. Spectra taken during the first phase of the reaction suggest that the spectrum of the intermediate must be quite similar to that of starting material **7**. Probable reaction paths are shown in Scheme II where the alternatives are reversion to the imidazolium species (path A), hydration and ring opening, or immediate ring opening (path B). It is not obvious just which of these possibilities would be more likely since both involve poor leaving groups which would require protonation prior to or during elimination, i.e., specific- or general-acid catalysis. If path A is favored then the intermediate in the hydrolysis of **7** will be **1** or **2** again whereas if path B obtains then the intermediate must be **8** or **9** (neither of which would be predicted necessarily to accumulate either!). Note that path B would also be a possible route of breakdown of **2** in Scheme I.

The decision between paths A and B was made in this case on the basis of the results of the hydrolysis of **7** at pH 6 in the presence of 0.1 M imidazole, the latter added as a nucleophile to trap **1**. It is argued that if path A occurs then sufficiently high concentrations of imidazole should react with **1** to give *N*-carbethoxyimidazole which would hydrolyze to imidazole and yield no cleavage products. On the other hand, path B should lead to cleavage irrespective of whether imidazole was present or not. The extent of cleavage in this experiment was estimated by the yield of **3** by absorbance at 235 nm after subsequent addition of Tris as described in the Experimental Section; *N*-carbethoxyimidazole is rapidly converted to imidazole under these conditions. Since no 235-nm absorbing material was produced in this experiment (although a quantitative yield of **3** was obtained in the absence of imidazole), it is concluded that path A of Scheme II occurs; i.e., under the prevalent conditions the O leaving group of **7** is lost rather than N. This is in accord with what is observed in the case of amide acetals[13] where, unless the nitrogen atom is protonated, C–O cleavage is favored. Also in perhaps closer analogy, the hydrolysis of the ethanol adduct of the *N,O*-trimethylenephthalimidinium cation proceeds via C–O cleavage to yield the phthalimidinium ion.[14]

Hence both the DEP/imidazole reaction and the hydrolysis of **7** proceed by way of an intermediate that accumulates in solution. The similarity of the spectra of the intermediates and the reaction paths of the two reactions argues for the identity of these intermediates. Further there is kinetic consistency between the two reactions with respect to the behavior of the intermediates. If they are indeed one and the same, then the rate constant for conversion of the intermediate derived from **7** into **3** should be the same as that observed in the DEP/imidazole reaction. In the latter case the rate constant is immediately available since at high

Table II. Estimates of the Rate Constants of Scheme I at pH 6.0

	rate constant[a]	source
k_1	$5\ s^{-1}\ M^{-1}$	ref 16; 25 °C
k_{-1}	$10^{-7}\ s^{-1}$	ref 16; 25 °C
$k_2k_3/(k_{-2}+k_3)$	$1.0\ s^{-1}\ M^{-1}$	this work; estimated from rates of appearance of 2
k_{-3}	$1.9 \times 10^{-3}\ s^{-1}$	this work; assumes that the rate of elimination of H_2O from 2 is the same as that of EtOH from 7
k_4	$6.4 \times 10^{-3}\ s^{-1}$	this work; directly measured
k_5	$4 \times 10^{-5}\ s^{-1}$	ref 5

[a] The rate constant for DEP hydrolysis under these conditions for comparison would be around $6 \times 10^{-4}\ s^{-1}$.[8]

DEP concentration complete conversion into the intermediate is achieved (see above) but in the former case an A → B → C situation is present (eq 1, where I is the intermediate). If I is the

$$7 \xrightarrow{k_a} \mathrm{I} \xrightarrow{k_b} 3 \qquad (1)$$

same intermediate as in the DEP/imidazole reaction, then it should be possible to fit the data of Figure 5 by assigning k_b equal to the observed rate constant for conversion of the intermediate in the latter reaction to **3**, given in Table I and using k_a and ϵ_1 as variable parameters. The value of ϵ_1 derived here should be the same as that measured directly in the DEP/imidazole reaction at high DEP concentrations. In fact the data of Figure 5 can be readily fitted by this method, giving $k_a = 2.17 \times 10^{-3}\ s^{-1}$ and $\epsilon_1 = 6700$. The latter compares well with that estimated from the DEP/imidazole reaction and with the directly measured ϵ_{240nm} of **7** (6600). This agreement provides strong evidence that it is the same intermediate observed in the reactions of Schemes I and II (path A). Finally it seems likely that the observed intermediate is the tetrahedral adduct **2**, since we have now shown that such species can be isolated (although **2** itself has not been). Certainly also the absorbance spectrum of the intermediate agrees well with that of **7**.

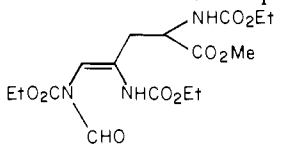
Rate constants for the ring-opening reactions of **2**, **7**, **5b**, and **5c** in 0.1 M phosphate buffer at pH 6 are collected in Table I. It is assumed in the benzimidazole cases **5b** and **5c** that the analogue of path A of Scheme II obtains so that the rate-determining step in the ring opening of **5c** must be ethanol elimination since the conversion of **5b** to **6b** is much faster than that of **5c**. It is noticeable that ethoxide/ethanol elimination from **7** is some 30 times faster than from **5c**. This must reflect the relative stabilities of the product aromatic cations with respect to their ethanol adducts. The rates of ring-opening of the subsequent hydroxy adducts **2** and **5b** are quite comparable. Details of the reactions of **2** and its analogues in aqueous solution will be further investigated.

For a conclusion of this section then, it seems likely that Scheme I is a good description of the DEP/imidazole reaction. Estimates for the rate constants at pH 6 in 0.1 M phosphate buffer are given in Table II. At low DEP concentration, acylation of 1-carbethoxyimidazole is rate determining with the accumulation of 1-carbethoxyimidazole in solution while at high DEP concentration the unimolecular (general base catalyzed probably[15]) decomposition of the 2-hydroxy-4-imidazoline intermediate **2** becomes rate-determining. At pH 6.0 neither the rate nor yield of cleavage (the latter essentially quantitative) can be increased by DEP concentrations higher than about 10 nM. At higher pH the reversion to rate-determining acylation occurs at higher initial DEP concentrations because of the competing hydrolysis of DEP. There is no evidence at pH 6.0 for the buildup in solution of significant concentrations of the diacylimidazolium ion intermediate **1**.

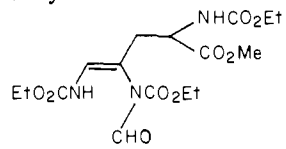
Reaction of DEP with Histidine Derivatives. As reported earlier[5] spectral changes accompanying the reaction of DEP with several histidine derivatives, including small peptides, are analogous to

(13) R. A. McClelland, *J. Am. Chem. Soc.*, **100**, 1844 (1978).
(14) N. Gravitz and W. P. Jencks, *J. Am. Chem. Soc.*, **96**, 507 (1974).
(15) N. Gravitz and W. P. Jencks, *J. Am. Chem. Soc.*, **96**, 489 (1974).
(16) W. B. Melchior, Jr., and D. Fahrney, *Biochemistry*, **9**, 251 (1970).

those observed with imidazole, i.e., formation of a product of high extinction coefficient with maximal absorption around 220 nm. Further studies of this reaction have been carried out by using histidine, histidine methyl ester, *N*-acetylhistidine, and glycylhistidinylglycine. In the case of histidine methyl ester a preparative reaction using the same procedure as for imidazole (see Experimental Section) yielded a mixture of the expected cleavage products **13** and **14** whose absorption spectrum (λ_{max} = 222 nm, ϵ = 19,000) resembled that of **3**. Further reaction at pH 7.5 yielded a product (λ_{max} = 228 nm, ϵ = 18,300) which is presumably the deformylated material analogous to **4**. Products similar to these have been isolated previously.[6,7]



13



14

The reaction of histidine methyl ester (0.1 mM) in aqueous solution (0.1 M phosphate, pH 6.0) with DEP (10 mM) yielding the above products did not, unlike that of imidazole, go to completion, since further DEP addition gave additional production of **13** and **14**. Apparently here the acylation step is rate determining even at 10 mM DEP. Measurement of cleavage rates under these conditions (rate of appearance of the 222-nm absorbing product) suggested that the overall cleavage reaction of the histidine derivatives, all of which mentioned above yielded similar rates, were some 10–15 times smaller than that of imidazole. The lower pK_a of the imidazole ring in histidine could be one factor contributing to this difference as could be the steric hindrance provided by the side-chain to acylation at the N^{π} position. This lower reactivity of histidine derivatives presumably explains the lower than originally expected yields of cleavage products seen on treatment of the proteins ribonuclease and liver alcohol dehydrogenase with DEP.[5] Approaches to circumvent this problem are being investigated.

Experimental Section

General Data. Melting points were determined on a Mel-Temp capillary melting point apparatus and are uncorrected. Infrared spectral data were obtained from a Perkin-Elmer Model 137 spectrophotometer, nuclear magnetic resonance spectra from a Varian Model A-60A spectrometer, using tetramethylsilane as an internal standard (δ 0), and mass spectra from a Hitachi Perkin-Elmer Model RMU-6L mass spectrometer. Elemental analyses were performed by Galbraith Laboratories (Knoxville, Tenn.). UV absorption spectra were obtained from a Cary 14 or a Cary 219 recording spectrophotometer, and spectrophotometric rate constants were determined by using either these instruments or a Gilford 2400 spectrophotometer; in each case the cell compartment was thermostated at 30.0 °C with a circulating water bath.

Materials. Imidazole (Sigma) was recrystallized twice from ethyl acetate before use. Benzimidazole (Aldrich) was recrystallized three times from water and once from ethyl acetate. Diethyl pyrocarbonate and ethyl chloroformate were obtained from Aldrich Chemical Co., while benzyl chloroformate and the histidine derivatives were purchased from Sigma Chemical Co. These latter reagents were used without further purification. Acetonitrile was distilled from P_2O_5 and stored over molecular sieves.

***N*-Carbethoxyimidazole.** The procedure of Staab[17] was followed. After the reaction mixture had stirred for 20 h, the precipitated imidazole hydrochloride was filtered off and the solvent was removed by rotary evaporation. The resulting liquid was distilled and the fraction boiling at 96–98 °C (12 torr) was collected (lit. bp 99–100 °C (12 torr)). The identity of this compound was confirmed by NMR and UV spectra.

***N,N'*-Dicarbethoxy-*N*-formyl-1,2-diaminoethene (3) and *N,N'*-Dicarbethoxy-1,2-diaminoethene (4).** These compounds were obtained in two ways, either by reaction of imidazole with DEP in dilute aqueous solution or in a two-phase mixture analogous to that employed by Patchornik et al.[11]

(i) In aqueous solution. Imidazole, 0.34 g (5 mmol), was dissolved in 2.5 L of 0.1 M phosphate buffer at pH 6.0 and at room temperature and 6.5 mL of DEP (40 mmol) added with stirring. The reaction mixture was then stirred for between 4 and 15 h after which the reaction was stopped by chloroform extraction of the products. The shorter reaction times yielded mainly **3** while the longer times yielded **4**. The chloroform extract was dried over $MgSO_4$ and the chloroform evaporated under reduced pressure, giving an oil. An NMR spectrum at this stage showed the absence of imidazole and the presence only of **3** and **4**, i.e., essentially quantitative conversion. Trituration of the oil with 3/1 benzene/hexane gave a colorless crystalline solid (**4**) which, after recrystallization from 1/1 benzene/hexane, had mp 133–135 °C. Evaporation of the triturate gave an oil (**3**) which could be purified by short-path distillation. The properties of **3** are as follows: IR (neat) 3400, 2900, 1650–1740 (br) cm^{-1}; UV (H_2O) λ_{max} 215 nm (ϵ 23 900); NMR (C^2HCl_3) δ 9.0 (5, 1, CHO), 6.45 (dd, J = 7, 9.5 Hz, 1, NH—C*H*═), 5.20 (d, J = 7 Hz, 1, >N—CH═), 4.35 (q, J = 7 Hz, 2, –CH_2–), 4.7 (q, J = 7 Hz, 2, –CH_2–), 1.38 (t, J = 7 Hz, 3, –CH_3), 1.28 (t, J = 7 Hz, 3, –CH_3); mass spectrum, *m/e* (relative intensity, %) 230 (55, M^+), 202 (100). Anal. Calcd for $C_9H_{14}N_2O_5$: C, 46.96; H, 6.09; N, 12.17. Found: C, 46.96; H, 6.10; N, 12.62. The properties of **4** are as follows: IR (KBr) 3300, 2900, 1660–1720 (br) cm^{-1}; UV (H_2O) λ_{max} 235 nm (ϵ 22 800); NMR (C^2HCl_3) δ 7.70 (br s, 2, NH), 5.67 (d, J = 7 Hz, 2, –NH—C*H*═), 4.08 (q, J = 7 Hz, 4, –CH_2–), 1.14 (t, J = 7 Hz, 6, –CH_3); mass spectrum, *m/e* (relative intensity, %) 202 (100, M^+). Anal. Calcd for $C_8H_{14}N_2O_4$: C, 47.53; H, 6.93; N, 13.86. Found: C, 46.94; H, 6.77; N, 13.82.

(ii) Ethyl chloroformate was added with stirring to imidazole dissolved in a 1/1 benzene/water mixture at pH 8 and at 0 °C as described by Patchornik et al.[11] Work-up as above after about 1 h yielded mainly **3** while longer reaction times yielded progressively more **4**.

Conversion of pure **3** to **4** could be achieved by stirring a suspension of **3** overnight in aqueous buffer (pH 6 or above); the product could then be isolated by filtration, drying, and recrystallization.

1,3-Dicarbobenzoxy-2-hydroxybenzimidazoline (5a). The general method of Patchornik et al.[11] was followed. The product, a white solid, was recrystallized from 50% hexane/benzene to achieve a yield of 62% with mp 115–116 °C (lit.[11] 115–117 °C): IR (KBr) 3375, 3010, 2950, 1720, 1680, 1610, 745 cm^{-1}; NMR (C^2HCl_3) δ 7.6–6.8 (m, 6, –C*H*OH, benzimidazole Ar–H), 7.25 (s, 10, benzyl Ar–H), 5.20 (s, 4, –CH_2Ph); mass spectrum, *m/e* (relative intensity, %) 404 (0.5, M^+), 314 (0.5), 296 (2), 269 (0.3), 252 (4), 225 (2), 224 (8), 134 (7), 107 (26), 91 (100).

***N,N'*-Dicarbobenzoxy-*N*-formyl-1,2-diaminobenzene (6a).** This compound, a colorless oil, was prepared from **5a** by addition of 1.1 mol % of triethylamine to a solution of **5a** in acetonitrile followed, after 5 min, by evaporation to dryness: IR (neat) 3380, 3060, 2975, 1735, 1700, 1600, 1365, 760, 740, 685 cm^{-1}; NMR (CH_3CN) δ 9.20 (s, 1, CHO), 7.9–6.8 (m, 4, benzimidazole Ar–H), 7.12 (s, 5, benzyl Ar–H), 7.06 (s, 5, benzyl Ar–H), 4.93 (s, 2, –CH_2Ph), 4.85 (s, 2, –CH_2Ph); mass spectrum, *m/e* (relative intensity, %) 404 (0.5, M^+), 314 (0.5), 296 (5), 269 (1), 252 (5), 225 (21), 224 (11), 134 (1), 107 (51), 91 (100). Anal. Calcd for $C_{23}H_{20}N_2O_5$: C, 68.30; H, 5.00; N, 6.93. Found: C, 68.13; H, 5.20; N, 6.95.

1,3-Dicarbethoxy-2-hydroxybenzimidazoline (5b). The procedure of Patchornik et al.[11] was followed by using ethyl chloroformate rather than benzyl chloroformate. The product was recrystallized from acetonitrile in 81% yield: mp 122–124 °C; IR (KBr) 3350, 2960, 2900, 1720, 1675, 1600, 750 cm^{-1}; UV ($CHCl_3$) λ_{max} 291 nm (ϵ 3500), 285 (4300), 246 (14 900); NMR (C^2HCl_3, 10% [$^2H_6$]Me_2SO) δ 8.0–6.9 (m, 5, C*H*OH, benzimidazole Ar–H), 4.40 (q, J = 7 Hz, 4, –OCH_2–), 1.40 (t, J = 7 Hz, 6, –CH_3); mass spectrum, *m/e* (relative intensity, %) 280 (38, M^+), 208 (47), 190 (36), 162 (30), 160 (39), 134 (100), 118 (43). Anal. Calcd for $C_{13}H_{16}N_2O_5$: C, 55.70; H, 5.77; N, 10.22. Found: C, 55.99; H, 5.76; N, 9.89.

Treatment of **5b** in [$^2H_3$]MeCN/[$^2H_6$]Me_2SO (4/1) with catalytic amounts of base as for **5a** gave a material whose NMR spectrum identifies it, by analogy with **6a**, as *N,N'*-dicarbethoxy-*N*-formyl-1,2-diaminobenzene (**6b**): δ 8.93 (s, 1, CHO), 8.0 (br s, 1, N–H), 7.8–6.7 (m, 4, ArH), 4.10 (q, J = 7 Hz, 2, OCH_2), 3.99 (q, J = 7 Hz, 2, OCH_2), 1.19 (t, J = 7 Hz, 3, CH_3), 1.15 (t, J = 7 Hz, 3, CH_3). UV (H_2O): λ_{max} 271 nm (ϵ 800), 228 (10 200).

1,3-Dicarbethoxy-2-ethoxybenzimidazoline (5c). The use of DEP rather than ethyl chloroformate in the procedure of Patchornik et al.[11] yielded this compound (63%), mp 116–118 °C, after recrystallization from 50% hexane/benzene: IR (KBr) 2965, 2905, 1720, 1600, 1195, 1020, 745 cm^{-1}; UV (H_2O) λ_{max} 290 nm (sh) (ϵ 3100), 282 (3400), 243 (13 200); NMR (C^2HCl_3) 7.55–6.85 (m, 5, HCO–, benzimidazole Ar–H), 4.33 (q, J = 7.0 Hz, 4, –CO_2 CH_2–), 3.78 (q, J = 7.5 Hz, 2, –OCH_2–), 1.30 (t, J = 7.0 Hz, 6, –$CO_2CH_2CH_3$), 1.19 (t, J = 7.5 Hz, 3, –OCH_2CH_3); mass spectrum, *m/e* (relative intensity, %) 308 (22, M^+), 262 (100), 235 (2), 190 (23), 175 (12), 163 (14), 147 (59), 118 (53). Anal. Calcd for $C_{15}H_{20}N_2O_5$: C, 58.42; H, 6.55; N, 9.09. Found: C, 58.19; H, 6.72; N, 9.00.

1,3-Dicarbethoxy-2-ethoxy-4-imidazoline (7). This compound was prepared from imidazole and DEP in a way analogous to that in which

(17) H. A. Staab, *Justus Liebigs Ann. Chem.*, **609**, 83 (1957).

5c was obtained. In this case however a pH of 6 rather than 8 was maintained since a trial run at the higher pH showed that much 3 as well as 7 was obtained under the latter condition. Imidazole (2.0 g, 0.0294 mol) was dissolved in 60 mL of water in a 500-mL Erlenmeyer flask equipped with a mechanical stirrer and the pH adjusted to 6 with 1 M hydrochloric acid. After the solution was cooled to 0 °C, 10.6 g (0.0655 mol) of DEP in 60 mL of benzene was added in a slow stream with stirring. The reaction mixture was then maintained at pH 6 by addition of 1 M sodium hydroxide and at 0 °C while being stirred over 1 h. The benzene layer was then separated from the aqueous which was extracted further with two 20-mL portions of benzene. The combined benzene solutions were dried over sodium sulfate, filtered, and evaporated to dryness. The oily residue was distilled under vacuum in a short-path apparatus, yielding 7 (45%): bp 107 °C (0.1 torr); IR (neat) 3075, 2900, 2875, 1705, 1605 cm^{-1}; UV (MeCN) λ_{max} 240 nm (ϵ 6600); NMR (C^2HCl_3) δ 6.60 (s, 1, HCO), 6.20 (s, 2, CH=), 4.20 (q, J = 7.0 Hz, 4, $-CO_2CH_2-$), 3.73 (q, J = 6.5 Hz, 2, $-O-CH_2-$), 1.29 (t, J = 7.0 Hz, 6, $-CO_2CH_2CH_3$), 1.18 (t, J = 6.5 Hz, 3, $-OCH_2CH_3$); mass spectrum, m/e (relative intensity, %) 258 (4, M^+), 213 (23), 140 (38), 97 (16), 95 (17), 69 (35), 68 (100). Anal. Calcd for $C_{11}H_{18}N_2O_5$: C, 51.14; H, 7.04; N, 10.85. Found: C, 51.46; H, 6.94; N, 11.00.

Methyl 2,4-Bis(carbethoxyamino)-5-(*N*-formylcarbethoxyamino)-pent-4-enoate (13) and Methyl 2,5-Bis(carbethoxyamino)-4-(*N*-formylcarbethoxyamino)pent-4-enoate (14). These compounds were prepared from histidine methyl ester and DEP in the same way as the imidazole analogue 3 (method i). The reaction was followed spectrophotometrically by taking aliquots for absorbance measurement (after dilution) at 222 nm. When maximal absorbance was achieved, the reaction mixture was extracted with chloroform. Evaporation of the dried chloroform extracts yielded an oil whose NMR spectrum indicated the presence of a histidine derivative and of two formyl compounds. Chromatography on silica gel in 9/1 methylene chloride/ethyl acetate yielded 34% of a 40/60 mixture of 13 and 14 as a viscous oil. The properties of this mixture were as follows: IR (neat) 3270, 2940, 1685 (br), 772 cm^{-1}; NMR δ 9.17, 9.11 (s, 1, CHO, 13, 14), 6.75 (d, $J \simeq 9$ Hz, NH—*CH*=, 14), 6.2 (br s, 1, NH, 13, 14), 5.53 (s, 1, >N—CH=, 13), 4.37 (q, 2, $N(CHO)CO_2CH_2CH_3$, 13, 14), 4.13 (q, 4, $NHCO_2CH_2CH_3$, 13, 14), 3.76, 3.68 (s, 3, CO_2CH_3, 13, 14), 3.2-2.5 (m, 2, CH_2—CH, 13, 14), 1.37 (t, 3, $N(CHO)CO_2CH_2CH_3$, 13, 14), 1.25 (t, 6, $NHCO_2CH_2CH_3$, 13, 14); mass spectrum, m/e (relative intensity, %) 403 (4, M^+), 375 (5), 314 (4), 286 (50), 243 (100), 215 (78), 169 (42). Anal. Calcd for $C_{16}H_{25}N_3O_9$: C, 47.64, H, 6.25; N, 10.42. Found: C, 47.78; H, 6.40; N, 10.24.

Reactions of DEP with Imidazole and Histidine Derivatives in Dilute Aqueous Solution. Typically these were studied spectrophotometrically in reaction mixtures containing the imidazole derivative (0.01-0.1 mM) in an appropriate buffer solution. Reactions were initiated by addition of a small aliquot of DEP in acetonitrile solution to the aqueous solution in a thermostated cuvette. The change in spectrum with time was then monitored. Yields of imidazole cleavage products obtained from reactions studied as above were determined by the amount of 4 produced, as follows. After the DEP reaction was judged complete by the absence of further spectral change, an aliquot of 3.24 M Tris solution was added to the reaction mixture, giving a final solution containing 0.2 M Tris at a pH between 8 and 10. Under these conditions all 3 is converted into 4 and all *N*-carbethoxyimidazole back to imidazole. The total extent of imidazole cleavage that had occurred could then be determined by the remaining absorbance at 234 nm, a measure of 4 concentration.

Acknowledgment is made to Wesleyan University and to the National Institutes of Health for support of this research.

Mechanism of Reactions of *N*-(Methoxymethyl)-*N*,*N*-dimethylanilinium Ions with Nucleophilic Reagents[1]

Barry L. Knier and William P. Jencks*

Contribution No. 1326 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. *Received January 14, 1980*

Abstract: The prediction that the oxocarbonium ion derived from formaldehyde should have a "lifetime" of $\sim 10^{-15}$ s that gives rise to an enforced preassociation or concerted reaction mechanism has been tested by examining the reactions of *N*-(methoxymethyl)-*N*,*N*-dimethylanilinium ions in water in the presence of added nucleophilic reagents. These compounds undergo well-behaved second-order reactions with nucleophiles and give the amount of substitution product that is expected from the rate increase in the presence of nucleophile. Structure-reactivity correlations exhibit behavior intermediate between that expected for S_N2 (Swain-Scott) and carbonium ion (N^+) reactions. The small values of s = 0.3 and β_{nuc} = 0.14 and large values of β_{lg} = -0.7 to -0.9 indicate a transition state that can be described either as an open transition state for S_N2 displacement or as an oxocarbonium ion that is stabilized by weak interactions with the entering and leaving groups. Secondary α-deuterium isotope effects for the second-order reactions range from $(k_H/k_D)/D$ = 0.99 for fluoride ion to 1.18 for iodide ion. Solvolysis and the second-order reaction with *n*-propylamine exhibit values of $\Delta S^{\ddagger}$ = -1.2 and -2.1 cal K^{-1} mol^{-1}, respectively, and display similar changes in rate in mixed water-alcohol solvents. It is suggested that the lifetime of the carbonium ion species in the presence of nucleophiles is so short that the reaction mechanism must be concerted.

We have been interested in the extent to which reaction mechanisms, and transitions between reaction mechanisms, are determined by the lifetimes of species that might exist as intermediates in the reaction. A knowledge of these lifetimes provides a relatively simple approach to the vexing problem of the nature and meaning of "borderline", "intermediate", "merging", and "solvent-assisted" mechanisms.[2-7]

The cleavage of a series of substituted acetophenone ketals has been shown to proceed through oxocarbonium ion intermediates with significant lifetimes by trapping these intermediates with sulfite dianion; the rate of ketal cleavage is independent of sulfite concentration.[8] Such a stepwise mechanism and the absence of a second-order reaction with nucleophilic reagents are charac-

(1) Supported by grants from the National Science Foundation (No. BG-31740) and the National Institutes of Health (No. GM20888).
(2) Dewar, M. J. S. *Annu. Rep. Prog. Chem.* **1951**, *48*, 121. Doering, W. v. E.; Zeiss, H. H. *J. Am. Chem. Soc.* **1953**, *75*, 4733-4738.
(3) More O'Ferrall, R. A. *J. Chem. Soc. B* **1970**, 274-277.
(4) Sneen, R. A. *Acc. Chem. Res.* **1973**, *6*, 46-53. See also: McLennan, D. J. *Ibid.* **1976**, *9*, 281-287.
(5) Bordwell, F. G.; Mecca, T. G. *J. Am. Chem. Soc.* **1975**, *97*, 123-127, 127-131. Bordwell, F. G.; Wiley, P. F.; Mecca, T. G. *Ibid.* 132-136.
(6) Harris, J. M. *Prog. Phys. Org. Chem.* **1974**, *11*, 89-173.
(7) Bentley, T. W.; Schleyer, P. v. R. *Adv. Phys. Org. Chem.* **1977**, *14*, 1-67. *J. Am. Chem. Soc.* **1976**, *98*, 7658-7666. Schadt, F. L.; Bentley, T. W.; Schleyer, P. v. R. *Ibid.* **1976**, *98*, 7667-7674. Arnett, E. M.; Petro, C.; Schleyer, P. v. R. *Ibid.* **1979**, *101*, 522-526.
(8) Young, P. R.; Jencks, W. P. *J. Am. Chem. Soc.* **1977**, *99*, 8238-8248.

0002-7863/80/1502-6789$01.00/0