Reactions of Cyanate with Functional Groups of Proteins. II. Formation, Decomposition, and Properties of *N*-Carbamylimidazole*

George R. Stark

ABSTRACT: Carbamylimidazole, in equilibrium with imidazolium cyanate in water, functions very poorly or not at all as a carbamylating agent. At *p*H values near 8, 0.4 M imidazole accelerates neither the rate at which cyanate decomposes nor its rate of reaction with several amines; the rates of reaction with alanine and β-alanine are increased only slightly in the presence of 1.0 M imidazole. In the absence of cyanate, 0.2 M glycylglycine does not affect the rate at which carbamylimidazole decomposes. These results are discussed with particular reference to a recent report that the reactive serine of α-chymotrypsin reacts with cyanate under mild conditions. The apparent equilibrium constant for imidazolium cyanate $\rightleftharpoons$ *N*-carbamylimidazole, K_t, increases with increasing concentration of IM, the free-base form of imidazole. When the concentration of IM = 0.08 at 25°, K_t = 0.25, corresponding to $\Delta F°$ = 0.82 kcal/mole; $\Delta H°$ = −2.1 kcal/mole, and $\Delta S°$ = −9.9 entropy units at 25°. In the absence of cyanate and imidazole, the rate at which carbamylimidazole dissociates, determined by measuring its absorbancy at 230 mμ, is independent of *p*H above about 6 (half-time about 1 minute at 25°) but decreases at lower *p*H values. Such behavior is consistent with a mechanism in which carbamylimidazolium cation (pK_a' = 4.2 at 25°) is attacked by hydroxide ion.

Much impetus for the present work was provided by a recent report (Shaw *et al.*, 1964) that cyanate reacts rapidly with the same reactive serine residue in α-chymotrypsin that is phosphorylated by diisopropylphosphorofluoridate. Histidine has been shown to be an essential part of the active site of chymotrypsin (Schoellman and Shaw, 1963; Ong *et al.*, 1964) and the possibility that acylation of the hydroxyl group of serine in this enzyme might proceed by way of an *N*-acylimidazole has been discussed often. Therefore, in considering mechanisms for the reaction of chymotrypsin with cyanate, one needs to know whether or not carbamylimidazole is formed from aqueous imidazolium cyanate and, if formed, whether it is capable of transferring the carbamyl group. Carbamylimidazole is *not* an efficient carbamyl atingagent, in contrast to *N*-acylimidazoles, which are very good acylating agents (see, for example, Jencks and Carriuolo, 1959b). This finding is important not only in clarifying the reaction of cyanate with the aliphatic hydroxyl group of serine in chymotrypsin but also is fundamental to an understanding of mechanisms by which cyanate can react with other functional groups in proteins.

Experimental Procedure

Interaction of Cyanate and Imidazole. When a solution containing an appreciable proportion of imidazole as the free base is added to a solution of KNCO, protons are taken up because the equilibrium between IMH^+ and IM is disrupted by formation of *N*-carbamylimidazole.[1] The amount of acid required to titrate the system back to the initial *p*H is a measure of the amount of $IMCONH_2$ formed. An expression for K_t, the apparent equilibrium constant, in terms of the quantities that are measured experimentally

$$K_t = \frac{[IMCONH_2]}{[IMH^+][NCO^-]} = \frac{\Delta(K_{im} + [H^+])^2}{[H^+][NCO^-][K_{im}IM_T - \Delta(K_{im} + [H^+])]} \quad (1)$$

can be derived from the relationship $\Delta/V = [IMCONH_2][IM]/([IM] + [IMH^+])$ and from the equations for equilibrium and conservation. Here, Δ is the number of millimoles of acid required to restore the *p*H to its original value and V is the final volume; IM_T is the total number of millimoles of imidazole added initially, and K_{im} is the acidic dissociation constant of imidazole. Since K_{NCO}, the acidic dissociation constant of cyanic acid, equals $10^{-3.73}$ at 27° (Lister, 1955), the approximation that $[NCO^-] = [NCO^-] + [HNCO]$ is excellent above *p*H 6 and has been used.

* From the Department of Biochemistry, Stanford University School of Medicine, Palo Alto, Calif. *Received August 17, 1964.* For paper I of this series, see Stark (1964). Supported by grants from the National Institutes of Health, U.S. Public Health Service, and the National Science Foundation. Some preliminary studies were carried out at the Rockefeller Institute, New York City.

[1] The abbreviations used are: IMH^+ and IM for protonated and unprotonated imidazole, $IMCONH_2$ and IMH^+CONH_2 for unprotonated and protonated *N*-carbamylimidazole.

In a typical determination, imidazole (Eastman White Label, recrystallized from benzene, mp 89.5–90.5°) was dissolved in aqueous acetic acid. The *p*H of this solution could be varied by changing the concentration of the acid. The *p*H of a mixture of one volume of such an imidazole acetate buffer and five volumes of aqueous KCl was determined to within 0.005 unit with a Metrohm Combi-titrator (Brinkmann Instruments, Inc.) in a closed reaction vessel thermostated to ±0.1°. A solution of KNCO (Matheson Coleman and Bell, reagent grade, recrystallized from ethanol-water at 50°) was prepared freshly at a concentration equal to that of the KCl solution and 1.5 ml was introduced into the clean, dry vessel. The *p*H was adjusted automatically with a small volume of 0.115 M acetic acid to the same value given by the mixture of imidazole, KCl, and acetic acid; 300 μl of the imidazole acetate buffer was added, whereupon the *p*H immediately increased. It was readjusted automatically to the previous value within about 10 minutes by the addition of 0.115 M acetic acid. Thereafter, a relatively slow and linear addition of acid continued because of the slow decomposition of cyanate. Although the cyanate concentration did not change significantly, the values of Δ had to be corrected by a small amount to account for the protons consumed as a result of cyanate decomposition during the time required to readjust the *p*H. In control experiments, no change in *p*H was noted when 300 μl of a solution of potassium acetate and ethanol in water, at concentrations equal to those of imidazolium acetate and free imidazole, respectively, was added to 1.5 ml of KNCO. After three determinations, using the same solutions, the *p*H of the mixture of imidazole, KCl, and acetic acid was checked again. It seldom varied at all from the first value and never by more than 0.005 *p*H. The reading given by a standard buffer was constant to within 0.01 *p*H during a day's work.

An uncertainty of ±5–10% in the value of K_i arises because the increase in *p*H after addition of imidazole is small, since the resulting mixture is a strong buffer. Therefore, a small error in readjusting the *p*H is reflected by a large error in Δ. The range of *p*H values that could be studied was limited because Δ was too small to be determined accurately above *p*H 8.1, whereas cyanate decomposed too rapidly below *p*H 6.8.

Spectrum of Carbamylimidazole in Water and Temperature Dependence of K_i. A portion of a stock solution of imidazole, partially neutralized with HCl, was mixed with aqueous KCl and placed in a 2-mm cell; a solution of KNCO was placed in a second cell. The final concentrations of KNCO, KCl, and imidazole were the same. With both cells in the same light path, the spectrophotometer (Zeiss PMQ II) was set to 100% transmission, slit width 0.16–0.30 mm. The same stock solution of imidazole was mixed with KNCO in a third cell and KCl alone was placed in a fourth. The absorbancy of the contents of the last two cells in series was then determined. Temperature was controlled by means of a thermostated cell holder; the temperature of the solution was read directly, by use of a thermistor probe. It was possible to attain thermal equilibrium within 5 minutes after a 5° change in temperature.

Decomposition of Carbamylimidazole. This compound, synthesized according to Lowenstein (1956), gave the expected analyses for C, H, and N to within 0.4%. Crystals were pulverized and dissolved rapidly in phosphate buffers, ionic strength 0.6, that had been equilibrated to a known temperature. The solutions were transferred immediately to a cuvet in the thermostated cell compartment of a Cary spectrophotometer, Model 14, and the absorbancy at 230 mμ was recorded as a function of time.

Rates of Carbamylation. Chromatographically homogeneous samples of glycine, threonine, glycylglycine, alanine, and β-alanine were dissolved in 10 ml of a solution containing both KNCO and imidazole at 30° to give final concentrations of 2–4 mM. In the experiments with glycine, threonine, and glycylglycine, reaction mixtures with and without imidazole were identical, except that the concentration of imidazolium acetate, 0.041 M, was duplicated by potassium acetate and that of free imidazole, 0.36 M, by ethanol. The *p*H was held constant at 7.89 during the reaction by automatic addition of less than 0.1 ml of acetic acid. After appropriate intervals, small portions of the reaction mixture were pipetted into an excess of HCl to halt the carbamylation. The residue remaining after the solvent had been removed *in vacuo* was dissolved in *p*H 2.2 buffer and chromatographed on an amino acid analyzer (Spackman *et al.*, 1958). Each measurement of rate was performed in duplicate.

Results

Titrimetric Determination of K_i. The data of Table I reveal that K_i, calculated according to equation (1), is nearly constant at similar values of *p*H, and does not depend on the total concentration of either imidazole or cyanate. However, a reasonably good linear correlation can be obtained between the value of K_i and the concentration of unprotonated imidazole, as shown in Figure 1. Such an effect would be obtained either if imidazole were to catalyze the formation of carbamylimidazole or if imidazolium ions (or hydrogen ions) were to catalyze its decomposition. The former possibility is almost certainly correct since 0.1 M imidazolium ion has no effect on the rate at which carbamylimidazole decomposes (Table VII) and since this rate is independent of hydrogen ion concentration above *p*H 6 (Table V).

A few determinations of K_i were carried out at temperatures other than 25°, with the results shown at the bottom of Table I. Although K_i does not appear to vary very much with temperature (but see the spectrophotometric determination following), the *p*H of a single imidazolium acetate–KCl mixture without cyanate changed progressively from *p*H 7.51 at 20° to *p*H 7.22 at 35°, indicating that K_{im} is quite temperature dependent. This was confirmed by titrimetric de-

TABLE I: Determination of K_t, the Apparent Equilibrium Constant for the Imidazolium Cyanate–Carbamylimidazole Equilibrium.

Temperature (°C)	pH	$[IM_T]^a$ (M)	$[NCO^-]$ (M)	v^b (ml)	K_t^c (M^{-1})
25	8.075	0.305	0.609	0.112	0.38
25	8.035	0.316	0.316	0.069	0.42
25	7.640	0.293	0.585	0.181	0.34
25	7.595	0.308	0.308	0.119	0.40
25	7.565	0.319	0.159	0.063	0.39
25	7.400	0.304	0.304	0.129	0.37
25	7.150	0.286	0.571	0.190	0.28
25	7.150	0.151	0.602	0.107	0.30
25	7.110	0.157	0.314	0.071	0.37
25	6.950	0.226	0.436	0.135	0.27
25	6.860	0.293	0.585	0.157	0.24
25	6.820	0.308	0.308	0.099	0.28
25	6.800	0.319	0.159	0.057	0.30
20	7.510	0.306	0.306	0.124	0.35
30	7.310	0.306	0.306	0.121	0.34
35	7.220	0.306	0.306	0.115	0.32

[a] The total amount of imidazole, IM_T, was either 0.3 or 0.6 mmole in a total volume of about 2 ml. [b] The volume of 0.115 M acetic acid required to adjust the pH of the imidazole-cyanate mixture to the value given by a mixture of imidazole with an amount of KCl equal to that of the cyanate. [c] Calculated according to equation (1).

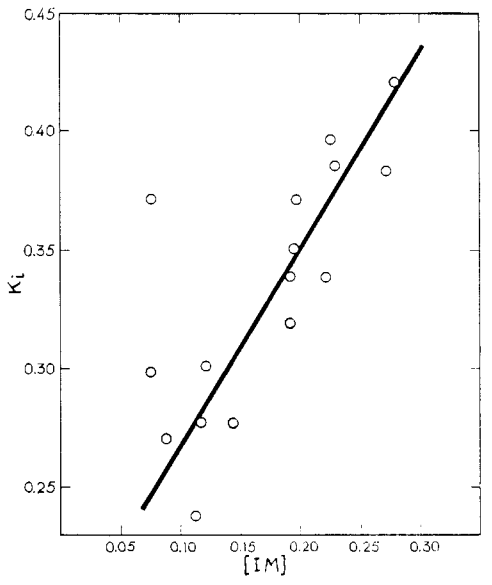


FIGURE 1: Dependence of K_t on the concentration of unprotonated imidazole. Values of K_t at 25° are taken from Table I; $K_{im} = 10^{-7.15}$ (Table II).

termination of K_{im} as a function of temperature at ionic strength 0.55. The values of K_{im} summarized in Table II, determined in duplicate at each temperature, have been used in all calculations.

Spectrum of Carbamylimidazole. *N*-Carbamylimidazole has a maximum absorbancy at 230 mμ in water,

TABLE II: pK of Imidazole as a Function of Temperature.[a]

Temperature (°C)	pK_{im}
20	7.27
25	7.15
30	7.08
35	7.00
40	6.90

[a] Ionic strength = 0.55.

ε = about 2000. Spectra at three values of pH are shown in Figure 2, and the extinction as a function of pH and concentration in Table III. The values of K_t used in calculating ε were derived from Figure 1. The apparent decrease in ε with decreasing concentrations of IM_T and NCO^-, well outside the limits of error in the spectrophotometric determination, is probably a reflection of the inaccuracy of deriving the values of K_t from Figure 1. Cyanate absorbs very little in the region where carbamylimidazole absorbs maximally, and no difference spectrum is obtained when cyanate at pH 7 is compared with cyanate at pH 10. Although both IMH^+ and IM do absorb in this region, the IM concentration does not change significantly when cyanate is added to a solution of imidazole and, although the decrease in IMH^+ is essentially equal to the increase

TABLE III: Extinction Coefficients for Carbamylimidazole.[a]

pH	[NCO⁻] or [IM$_T$] (M)	[IM] (M)	K_t[b] (M^{-1})	[IMCONH$_2$][c] (10^{-4} M)	ϵ (at 230 mμ)
7.16	0.100	0.050	0.225	13.55	2650
7.14	0.075	0.038	0.215	8.18	2490
7.14	0.060	0.030	0.209	5.44	2465
7.65	0.100	0.076	0.247	5.98	2195
7.63	0.075	0.056	0.230	3.77	2040
7.63	0.060	0.045	0.221	2.55	1890
7.94	0.120	0.103	0.270	4.59	2170
7.94	0.100	0.086	0.255	3.40	1860
7.93	0.087	0.075	0.246	2.65	1770

[a] Difference spectra at 25.0° were determined as described in the text. [b] Values are obtained from Figure 1. [c] Calculated from $[IMCONH_2] = K_t[NCO^-][H^+][IM_T]/([H^+](K_t[NCO^-] + 1) + K_{im})$, an expression derived from the definition of K_t and the equations for equilibrium and conservation. The symbols used are defined in the text and in footnote 1.

in carbamylimidazole, the change in concentration is small and the correction to the difference spectrum is not important above 225 mμ.

In contrast to its behavior in water or absolute ethanol, carbamylimidazole is stable in anhydrous tetrahydrofuran. In solvent freshly distilled from $FeSO_4$ a broad peak with a maximum at 226 mμ, ϵ = 3650, is the only feature of the ultraviolet spectrum. (Staab and Benz [1961] report that, in the same solvent, the *n*-butylamide of imidazole-1-carboxylic acid absorbs maximally at 222 mμ with ϵ = 4780.) The carbamylimidazolium cation, stable in water at *p*H 2 (*vide infra*), does not have a well-defined peak in the near ultraviolet. Absorbancy increases steadily from 250 to 210 mμ.

Temperature Dependence of K_t. Although it is not apparent that K_t is a function of temperature from the titrimetric data of Table I, the variation of K_t with T can be demonstrated quite readily by spectrophotometry, as shown in Table IV. In calculating K_t, the extinction at 230 mμ/mole of $IMCONH_2$ was assumed to be a constant. After the values at temperatures above 25° had been measured, the absorbancy at 25° was checked again and found to be unchanged in the last two determinations. However, in the first experiment, where the *p*H is lowest, the value redetermined at 25° was about 10% low, probably because some of the cyanate had decomposed. Plots of log K_t versus the reciprocal of absolute temperature were linear, with the slopes shown in the table.

Rate of Decomposition of Carbamylimidazole. Lowenstein (1956) has reported that carbamylimidazole decomposes to imidazolium cyanate in water. From his qualitative description, one has the impression that the decomposition is slow at room temperature. However, the present data indicate that this is so only if the solution is quite acidic. The absorbancy at 230 mμ of a dilute solution of *N*-carbamylimidazole in buffer decreases almost to zero within a few minutes,

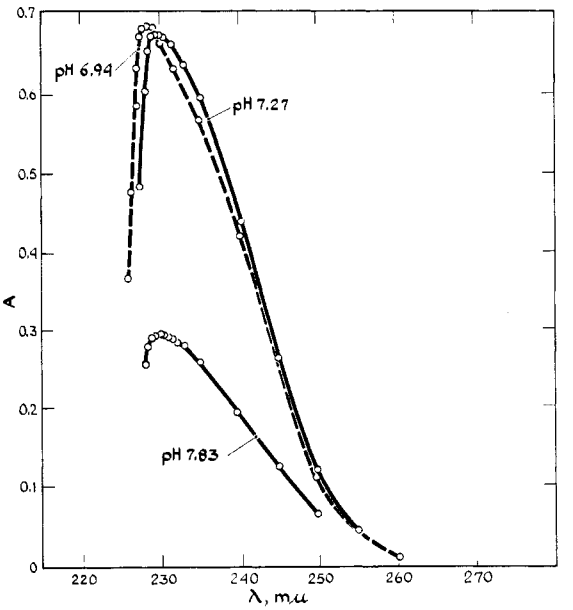


FIGURE 2: Spectra of *N*-carbamylimidazole. Imidazole and cyanate were each 0.1 M in a single 2-mm cuvet; spectra were determined by difference, as described in the text.

providing a convenient means of following the reaction. The data, summarized in Table V, reveal that the rate decreases below *p*H about 6 but is independent of *p*H in more alkaline solution. The rate of decomposition at *p*H about 12 (0.01 M NaOH in 0.5 M NaCl) was the same as that at *p*H about 7. Phosphate does not catalyze the decomposition of carbamylimidazole since the rate at *p*H about 7 is essentially unchanged in the absence of buffer.

TABLE IV: Spectrophotometric Determination of K_i as a Function of Temperature.

$[IM]_T$ or $[NCO^-]$ (M)	Temperature (°C)	pH^a	K_i^b (M^{-1})	$\frac{-d \log K_i}{d(1/T)}$
0.10	20.0	7.28	0.277	
0.10	24.8	7.16	0.250	
0.10	29.8	7.09	0.220	480
0.10	34.9	7.01	0.188[c]	
0.10	40.1	6.91	0.168[c]	
0.10	20.2	7.77	0.277	
0.10	25.0	7.65	0.250	
0.10	30.0	7.58	0.216	480
0.10	35.0	7.50	0.192	
0.10	40.0	7.40	0.171	
0.12	20.1	8.07	0.288	
0.12	25.0	7.95	0.250	
0.12	30.0	7.88	0.231	450
0.12	35.2	7.80	0.206	
0.12	40.0	7.70	0.185	

[a] The pH at 25° was determined experimentally; values at other temperatures were calculated from the data of Table II. [b] $K_i = \{[IMCONH_2]([H^+] + K_{im})/([NCO^-][H^+]([IM_T] - [IMCONH_2])\}$. The value of K_i at 25° was arbitrarily set at 0.25 in each determination. [c] Values probably low because of decomposition of cyanate.

TABLE V: Decomposition of Carbamylimidazole.

Temperature 25.0°		Temperature 39.8°	
pH^a	$-Slope^b$ (min^{-1})	pH^a	$-Slope^b$ (min^{-1})
3.260	0.026, 0.029	3.440	0.268, 0.289
3.580	0.054, 0.055	4.080	0.668, 0.724
4.040	0.116, 0.115	4.270	0.840, 0.804
4.320	0.160, 0.159	4.670	1.084, 1.058
4.695	0.208, 0.206	4.985	1.164, 1.138
4.975	0.245, 0.243	6.945	1.546, 1.500
5.400	0.281, 0.277		
6.280	0.295, 0.301		
6.945	0.307, 0.310		
7.840	0.296, 0.309		

[a] Phosphate buffers, about 0.2 M, ionic strength adjusted to 0.6 with NaCl. The pH was determined to within 0.005 unit at the temperature of the rate measurement. [b] Determined from linear plots of log (absorbancy) versus time.

If the rate law for the decomposition is written as rate = $k_{-1}[IMH^+CONH_2][OH^-]$, the slope of a plot of log (absorbancy) versus time will be given by

$$-\text{slope} = \frac{k_{-1}K_w}{2.3([H^+] + K_+)},$$

where K_w is the ion product of water and K_+ is the acidic dissociation constant of protonated carbamylimidazole. Taking the reciprocal of both sides yields

$$\frac{-1}{\text{slope}} = \frac{2.3[H^+]}{K_w k_{-1}} + \frac{2.3K_+}{K_w k_{-1}} \quad (2)$$

which predicts that a plot of -1/slope versus $[H^+]$ will be a straight line. (A straight line would be obtained also if the rate law were written rate = $k'_{-1}[IMCONH_2]$, but this expression is probably not in accord with the actual mechanism; see the Discussion.) Plots derived from the data of Table V are shown in Figure 3. From the slopes and intercepts of these it can be calculated that $K_+ = 10^{-4.21}$ at 25.0° and $10^{-4.00}$ at 39.8°. (The acidic dissociation constant of the acetylimidazolium cation is $10^{-3.6}$ [Jencks and Carriuolo, 1959a].)

The same data were used to calculate the values of k_{-1} recorded in Table VI. The dependence of k_{-1} on temperature is shown in Figure 4. Kinetic runs, in duplicate at each temperature, were made at pH 6.95, where the rate is not a function of pH.

Reactivity of Carbamylimidazole. The rates of carbamylation of glycine, threonine, and glycylglycine in 0.4 M KNCO at pH 7.89 and 30° are shown in Figure 5. There is no significant effect on any of these when 0.4 M imidazole is included in the reaction mixture at the same pH.

The specific rate constants for carbamylation of alanine and β-alanine in 1.0 M KNCO at pH 7.6 (calculated from the expression in the legend to Figure 5) are 8.56×10^{-3} and 5.74×10^{-3} M^{-1} min^{-1} at 30°. If 1.0 M imidazole is included, the constants increase

TABLE VI: Constants and Thermodynamic Parameters for the Imidazolium Cyanate–Carbamylimidazole Equilibrium.

Reaction	Constant at 25.0°	$\Delta H^{\circ a}$ (kcal/mole)	$\Delta F^{\circ b}$ (kcal/mole)	$\Delta S^{\circ c}$ (eu)
(1) $IMH^+ + NCO^- \rightleftharpoons IMCONH_2$	$K_t = 0.25$ M^{-1} [d]	− 2.1	+ 0.8	− 9.9
(2) $IM + HNCO + H_2O \rightleftharpoons IMH^+CONH_2 + OH^-$	$K_m = 10^{-6.94}$	− 2.5 [e]	+ 9.5	−40.1
		$\Delta H^{\ddagger}$ (kcal)	$\Delta F^{\ddagger}$	$\Delta S^{\ddagger}$ (eu)
(3) $IMH^+CONH_2 + OH^- \rightarrow H_2O + IM + HNCO$	$k_{-1} = 10^{9.61}$ M^{-1} min^{-1} (= $10^{10.00}$ at 39.8°)	+11.4 [f]	+ 6.8 [g]	+15.4 [h]
(4) $H_2O + IM + HNCO \rightarrow IMH^+CONH_2 + OH^-$	$k_1 = 10^{2.67}$ M^{-1} min^{-1}	+ 8.9 [i]	+16.3 [i]	−24.7 [i]

[a] $\Delta H^\circ = -2.3R(d \log K/d(1/T))$. [b] $\Delta F^\circ = -2.3\ RT \log K$. [c] $\Delta S^\circ = (\Delta H^\circ - \Delta F^\circ)/T$. [d] A representative value at [IM] ≅ 0.1; see Table I and Figure 1. [e] From equation (3), $\frac{d \log K_m}{d(1/T)} = [d \log \left(\frac{K_t K_{NCO} K_w}{K_+ K_{im}}\right)/d(1/T)]$. The following values were used: $d \log K_t/d(1/T) = 470$ (Table IV); $d \log K_{NCO}/d(1/T) \cong 0$ (Caramazza, 1958; Lister, 1955); $d \log K_w/d(1/T) = -2920$ (calculated from data in Hodgman, 1963); $d \log K_+/d(1/T) = -1310$ (from the values of K_+ at 25.0 and 39.8°); $d \log K_{im}/d(1/T) = -1690$ (from the data of Table II). [f] $\Delta H^{\ddagger} = -RT - 2.3R\ d \log k_{-1}/d(1/T)$ (see Figure 4). [g] $\Delta F^{\ddagger} = -2.3\ RT \log (k_{-1}h/k_B T)$, where h is the Planck constant and k_B is the Boltzmann constant. [h] $\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta F^{\ddagger})/T$. [i] Calculated from the values for reactions 2 and 3.

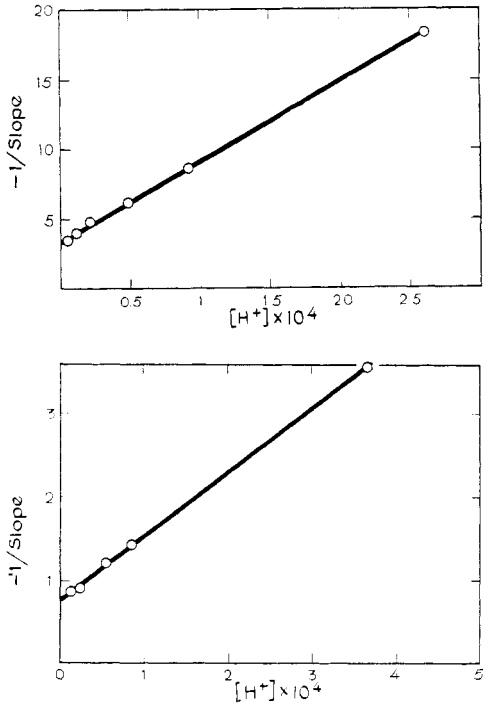


FIGURE 3: Dependence of the rate at which carbamylimidazole decomposes upon *p*H. Data of Table V are plotted according to equation (2). (a, upper) temperature = 25.0°; (b, lower) temperature = 39.8°.

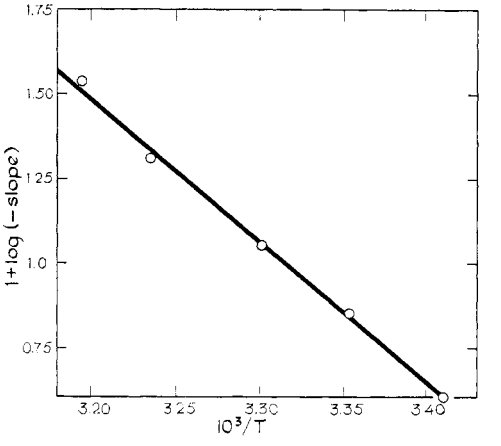


FIGURE 4: Dependence of the rate at which carbamylimidazole decomposes upon temperature. The slope referred to in the ordinate is that of a plot of log A_{230} versus time. The abscissa is the reciprocal of absolute temperature.

slightly, to 9.56×10^{-3} and 7.37×10^{-3} M^{-1} min^{-1} (average of duplicate experiments). Under these conditions, the concentrations of carbamylimidazole and HNCO are 0.14 M and 0.7×10^{-4} M, respectively. From these data, it can be calculated that, if carbamylimidazole reacts with the amino acids at all, it does so at a rate at least 5000 times slower than HNCO. The possibility remains that the small increase in rate of reaction with cyanate in the presence of a high concentration of imidazole is due solely to a small catalytic effect of unprotonated imidazole, just as that observed for the carbamylation of imidazole itself. That this explanation is probably correct is indicated by the data of Table VII: At *p*H 8.4, 0.2 M glycylglycine is without significant effect on the rate at which carbamylimidazole decomposes. The concentration of unprotonated peptide is about 0.12 M at this *p*H (pK'_a = 8.17, Greenstein and Winitz [1961]).

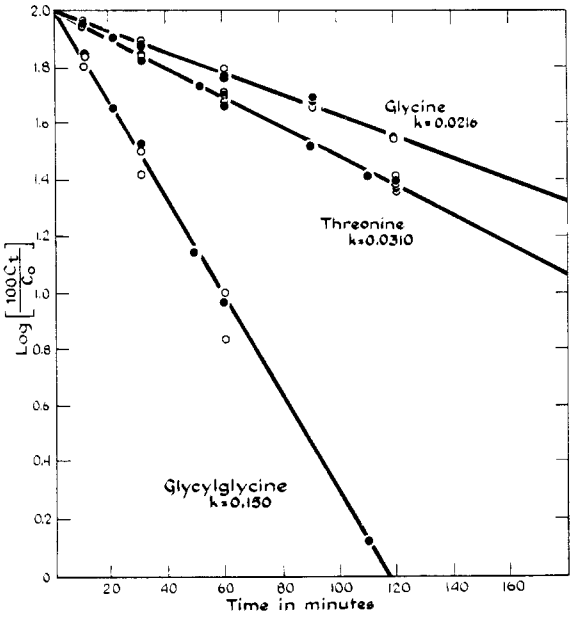


FIGURE 5: Rates of carbamylation in 0.4 M KNCO at *p*H 7.89 and 30°. ●, reaction in the absence of imidazole; ○, reaction in the presence of 0.4 M imidazole. In the function plotted on the ordinate, C_0 is the amount of amino compound at time = 0 and C_t the amount at time = t. Values of k, the specific rate constants in M^{-1} min^{-1}, are indicated in the figure and have been calculated from the expression $k = \{-2.3 \text{ slope } ([H^+] + K_a')/[NCO^-][H^+]\}$, where K_a' is the apparent acidic dissociation constant for the zwitterionic species of amino acid or peptide. Values of K_a' are from Greenstein and Winitz (1961).

TABLE VII: Decomposition of Carbamylimidazole in the Presence of Glycylglycine or Imidazole.[a]

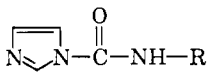
Addition	Concentration (M)	*p*H	−Slope (min^{-1})
Glycylglycine	0.2	8.40	0.318, 0.311[b]
Imidazole	0.1	5.90	0.304, 0.310

[a] Temperature 25°, ionic strength 0.6. [b] Rate of decrease determined at 240 mμ since glycylglycine interferes at 230 mμ.

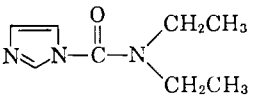
Discussion

It is informative to compare some of the properties of carbamylimidazole with those of acetylimidazole. Acetylimidazole and its cation are in equilibrium in water (pK'_a 3.6). Since the cation reacts with water at a much greater rate than the unprotonated form, and since both react rapidly with hydroxide ion, acetylimidazole is most stable at *p*H 7 (Jencks and Carriuolo, 1959a). Although carbamylimidazole is more stable at *p*H 5 than at *p*H 6, the rate of decomposition, in contrast to that of acetylimidazole, does not increase further with increasing *p*H. This fact and the failure of unprotonated glycylglycine to catalyze decomposition indicate that carbamylimidazole participates poorly or not at all in nucleophilic displacement reactions with bases. Further support for this idea is provided by the observation, made repeatedly during the titrimetric determinations of K_i, that imidazole does not catalyze the decomposition of cyanate. Such catalysis would be expected to result from a nucleophilic attack of hydroxide ion on the carbonyl carbon of carbamylimidazole, with concomitant formation of imidazole and carbamate.

The observation that carbamylimidazole is unstable is in accord with results of Staab (1957), who found that the anilide of imidazole-1-carboxylic acid dissociates to imidazole and phenyl isocyanate in aqueous tetrahydrofuran. More important, the finding that carbamylimidazole fails to function efficiently as a carbamylating agent is in complete agreement with results of Staab and Benz (1961): Carbamylimidazoles having the general formula

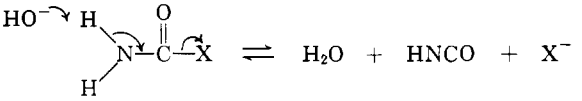


dissociate to imidazole and RNCO in chloroform and react with amines in this solvent to form substituted ureas. In contrast,



which cannot dissociate to form an isocyanate, is completely unreactive toward amines under similar conditions.

The decomposition of carbamylimidazole in water is similar to that of *S*-carbamyl compounds in that dissociation rather than hydrolysis occurs, except that the decomposition of *S*-carbamyl compounds *is* catalyzed by hydroxide ion (Stark, 1964). Both decompositions probably occur according to the following mechanism[2]:

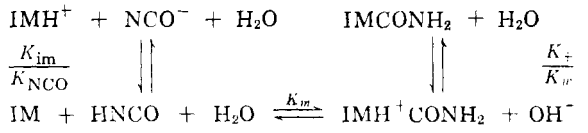


However, in the case of carbamylimidazole, the imidazole anion is such a poor leaving group that protonation is required before reaction can occur. The rate law for decomposition has been formulated as rate = $k_{-1}[IMH^+CONH_2][OH^-]$ rather than the kinetically equivalent expression rate = $k_{-1}'[IMCONH_2]$, be-

[2] Dr. Derek Smyth (personal communication) has found that cyanate reacts with the phenolic hydroxyl group of tyrosine to form a derivative that behaves similarly to *S*-carbamyl compounds; *O*-carbamyltyrosine is stable in acidic solution but decomposes rapidly at slightly alkaline *p*H.

cause it is unreasonable that IMH^+CONH_2 can be unreactive under conditions where $IMCONH_2$ eliminates IM^- rapidly.

The various equilibria and equilibrium constants are summarized by the following scheme:



where K_m is related to K_i, the apparent equilibrium constant for the overall process, by

$$K_m = \frac{K_i K_{NCO} K_w}{K_+ K_{im}} \quad (3)$$

In Table VI are summarized the rate and equilibrium constants, the standard free energies, enthalpies, and entropies for the equilibria, and the enthalpies and entropies of activation for reaction in both directions.

Shaw *et al.* (1964) have suggested that the inactivation of trypsin, chymotrypsin, and subtilisin upon treatment with cyanate is probably a result of carbamylation of the reactive serine of these proteins (demonstrated only for chymotrypsin) rather than carbamylation of amino groups. However, since the hydroxyl group of free serine is unreactive toward cyanate under conditions similar to the ones used by these authors,[3] a mechanism other than direct uncatalyzed reaction must be considered. A scheme in which cyanate reacts first with the imidazole of an essential histidine residue and is transferred subsequently to the hydroxyl of serine seems most unlikely in view of the evidence presented here that *N*-carbamylimidazole is unreactive as a carbamylating agent. Of course, it is always possible that the essential histidine residues of these enzymes have an unusual reactivity and that model experiments do not adequately represent the situation in the proteins. A much more likely explanation is that the nucleophilic character of the reactive serine hydroxyl is greatly enhanced, probably by general base catalysis involving the essential histidines.

[3] G. R. Stark, unpublished observation.

Acknowledgment

The expert technical assistance of Miss Anne R. Potts is gratefully acknowledged.

References

Caramazza, R. (1958), *Gazz. Chim. Ital. 88*, 308.
Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, New York, Wiley, pp. 486–488.
Hodgman, C. D. (ed.) (1963), Handbook of Chemistry and Physics, 44th ed., Cleveland, Ohio, Chemical Rubber Publishing Co., p. 1752.
Jencks, W. P., and Carriuolo, J. (1959a), *J. Biol. Chem. 234*, 1272.
Jencks, W. P., and Carriuolo, J. (1959b), *J. Biol. Chem. 234*, 1280.
Lister, M. W. (1955), *Can. J. Chem. 33*, 426.
Lowenstein, J. M. (1956), *J. Chem. Soc.*, 4667.
Ong, E. B., Shaw, E., and Schoellman, G. (1964), *J. Am. Chem. Soc. 86*, 1271.
Schoellman, G., and Shaw, E. (1963), *Biochemistry 2*, 252.
Shaw, D. C., Stein, W. H., and Moore, S. (1964), *J. Biol. Chem. 239*, PC671.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Staab, H. A. (1957), *Ann. 609*, 83.
Staab, H. A., and Benz, W. (1961), *Ann. 648*, 72.
Stark, G. R. (1964), *J. Biol. Chem. 239*, 1411.