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Reactions of Cyanate with Functional Groups of Proteins. III. Reactions with Amino and Carboxyl Groups*

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ABSTRACT: The velocity at which an amino acid or peptide is carbamylated can be estimated if its pK_A is known, since logarithms of the rate constants for reaction of KNCO with a series of such compounds are related linearly to the pK_A values of the amino groups. At *p*H 7 or below, the α-NH_2 groups of peptides and proteins (pK_A about 8) can be expected to react with cyanate about 100 times faster than the ϵ-NH_2 groups of lysine residues (pK_A about 10.7). As a consequence, α-NH_2 groups can be modified selectively under controlled conditions.

The conclusion that the reaction of amines with cyanate occurs by a mechanism involving the uncharged species and not the ions is supported strongly by present evidence that the relative rates of carbamylation by cyanate or ethyl isocyanate in water are the same for a series of ω-NH_2 acids. In addition to the expected carbamyl amino acids, 5-, 6-, and 7-membered lactams are formed by reaction of cyanate or ethyl isocyanate with γ-, δ-, or ϵ-NH_2 acids. These cyclic amides form from intermediate carbamylcarboxylates. Upon exposure of proteins to cyanate, amides, or esters might be formed similarly from favorably situated nucleophiles and the activated carboxyl group. Some general properties of carbamyl compounds are discussed, particularly those of carbamylphosphate that bear on the mechanism of action of ornithine and aspartate carbamyl transferases.

The work reported here began as a systematic attempt to extend our previous observation that glycylalanine reacts much more rapidly than alanine with cyanate at *p*H 8.0 (Stark and Smyth, 1963). Johncock *et al.* (1958) found little correlation between pK_A and rate of carbamylation for a series of 1° and 2° amines but, as shown in the present work, an excellent correlation can be obtained if consideration is limited to the relatively unhindered 1° amino groups of peptides and amino acids. The present results provide a basis for selective modification with cyanate of those protein and peptide amino groups that have low pK_A values. They also provide a way of examining the mechanism of the reaction.

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Experimental Procedures

Materials. Reagent grade KNCO was recrystallized from ethanol-water at a maximum temperature of 50°.

Ethyl isocyanate was redistilled through a fractionating column; small portions of the distillate at 60.0–60.5° were sealed in glass and stored at 4° until used. Peptides and amino acids from various commercial sources were all homogeneous on the amino acid analyzer (Spackman *et al.*, 1958).

Rates of Reaction with KNCO. Peptide or amino acid, final concentration about 2–4 mM, was placed in the closed reaction vessel of a *p*H-stat, a fresh solution of KNCO in water was added, and the mixture was stirred magnetically. The *p*H was adjusted to *p*H 8.00 ± 0.01 and kept at that value throughout the course of the reaction by the automatic addition of dilute acetic acid. Within a few minutes, after the temperature of the solution had reached 30 ± 0.1°, a known volume was removed (time = 0) and pipetted into at least 10 volumes of HCl, concentrated enough to destroy all the cyanate. Samples withdrawn later were prepared identically. Carbamyl amino acids and peptides were not hydrolyzed under these conditions. Excess acid was removed with a rotary evaporator, and the residue was dissolved in buffer and chromatographed on an amino acid analyzer. Since the amount of unreacted peptide or amino acid was determined chromatographically, it was often possible to follow the carbamylation of several compounds in a single reaction mixture. Straight lines were obtained from plots of the logarithm of the area under each peak versus time. The decomposition of cyanate at *p*H 8.0 and 30° is so slow (half-time about 500 hours) that no correction for this is needed. However, at concentrations of KNCO greater than 0.4 M a small correction was made to account for the increase in volume due to addition of acetic acid.

Carbamylation of L-*Alanyl*-L-*lysine*. The peptide, about 2×10^{-3} M in 0.2 M KNCO, was kept at *p*H 7.00 ± 0.01 and 30° in a *p*H-stat. After 1 hour, 2.5 ml of 0.5 M acetic acid was added to 5.0 ml of the reaction mixture and the solution was diluted to 10.0 ml. The extent of reaction at α- and ε-NH_2 groups was determined after heating a 1.0-ml portion of the solution and 1.0 ml of 12 M HCl to 100° for 1 hour. Under these conditions the unmodified peptide is hydrolyzed completely, the ε-ureido group of homocitrulline is almost completely stable (Stark *et al.*, 1960), and the α-carbamyl peptides are converted to the hydantoin of alanine plus lysine or homocitrulline (Stark and Smyth, 1963). The hydantoin was separated from amino acids by chromatography on Dowex 50-X8, hydrolyzed, and analyzed as described previously (Stark and Smyth, 1963). Amino acids were removed from the Dowex column with 1 M NH_4OH and analyzed separately.

Relative Rates of Reaction with Isocyanates. Since isocyanates are hydrolyzed rapidly, it is very difficult to determine their absolute rates of reaction with amines in aqueous solution. However, the *relative* rates for any two amines (X and Y) can be determined since these are proportional to the amounts of products formed and independent of the concentration of isocyanate:

$$\frac{\text{carbamyl X}}{\text{carbamyl Y}} = \frac{\text{rate}_x}{\text{rate}_y} = \frac{k_x(\text{X})(\text{RNCO})}{k_y(\text{Y})(\text{RNCO})}$$

Using K_A values for the amines, this equation can be rewritten:

$$\frac{k_x}{k_y} = \frac{\text{carbamyl X}}{\text{carbamyl Y}} \cdot \frac{K_Y}{K_X} \cdot \frac{(\text{YH}^+)}{(\text{XH}^+)}$$

The ratio $(\text{YH}^+)/(\text{XH}^+)$ is kept constant by keeping the *p*H well below the pK_A values of the amines and by keeping the concentrations of X and Y close to their initial values (small extents of reaction). Under these circumstances, determining the amounts of products that are formed allows one to calculate k_x/k_y. The argument is unchanged if more than two amines are present in the same reaction mixture. Accurate and reproducible results were not obtained when ethyl isocyanate was pipetted into a buffered, vigorously stirred mixture of amino acids, probably because reaction with the isocyanate is so rapid that most of the amine in a small portion of solution reacts before complete mixing can occur. This difficulty was circumvented by first adding the isocyanate to an imidazole buffer, then adding the mixture of amino acids. Isocyanates react rapidly with imidazole, and the *N*-carbamylimidazoles that are formed are not carbamylating agents themselves but dissociate readily to isocyanate and imidazole in aqueous solution (Staab and Benz, 1961; Stark, 1965). In a typical experiment, 2.0 ml of imidazole-HCl buffer, *p*H 7.0, 0.8 M, was equilibrated to 30.0 ± 0.1° with magnetic stirring. Fifty μl of ethyl isocyanate (or 50 mg of KNCO) was added, followed immediately by 2.0 ml of a solution 0.1 M in each amino acid, *p*H 7.0. After 2 hours at 30° (overnight for KNCO) the *p*H was 6.9. The mixture was diluted to 10.0 ml and 2.0 ml was applied to a column of Dowex 50-X8 (0.9 × 10 cm) in 0.1 M acetic acid. Effluent (400 ml) was collected, with 0.1 M acetic acid as eluent, evaporated to dryness, and dissolved in 3.0 ml of 0.4 M acetic acid. Two ml of this solution was applied to a column of Dowex 1-X8 (0.9 × 20 cm) in 0.4 M acetic acid. The first 25 ml and 25 through 125 ml of effluent were collected separately. Lactams appear in the first fraction and carbamylamino acids in the second.[1] Each fraction was evaporated to dryness, dissolved in 0.2 M NaOH, and hydrolyzed at 110° for 16 hours. The hydrolysates were prepared for analysis as described previously (Stark and Smyth, 1963) and chromatographed on an amino acid analyzer. The column (0.9 × 12 cm) was equilibrated to *p*H 3.28 (0.2 M Na^+) and run at this *p*H for 20 minutes, then changed to *p*H 5.28 (0.35 M Na^+). At a flow rate of 40 ml/hour, glycine, β-alanine, γ-aminobutyric acid, δ-aminovaleric acid, and ε-aminocaproic acid (40–50 ml) are completely resolved and emerge in the order given. No amino acids were found in the hydrolysates if the isocyanate was omitted, showing that the Dowex 50 columns are capable of completely removing the large excess of unreacted material.

[1] Carbamylglycine was not eluted completely from the Dowex 1-X8 columns under the conditions used and was determined by hydrolysis of a portion of solution before this step.

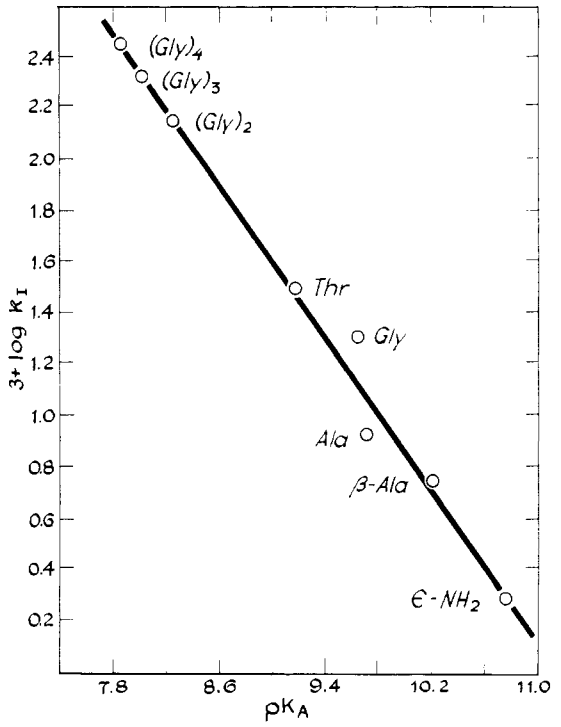


FIGURE 1: Dependence of rate constants upon pK_A for reactions of amino acids and peptides with KNCO at 30°. The data of Table I are plotted. Each point shown is the average of the values in the table. The line is that of the equation log k_I = 7.94 (±0.08) − 0.71 (±0.02) pK_A and was derived from a least-squares treatment of the individual experiments listed in Table I.

Recoveries were determined by reacting the amino acids with a large excess of isocyanate, then analyzing for the carbamylamino acids as before. Analysis of such reaction mixtures without prior chromatography or hydrolysis provided assurance that carbamylation was complete. Recoveries of 62–52% were determined for the *N*-ethylcarbamylamino acids and these values were used in correcting the results. The recoveries from carbamylamino acids were all about 95% and the results were not corrected.

Characterization of Some Reaction Products. α-Pyrrolidinone (the lactam from γ-aminobutyric acid) was prepared by reacting about 200 mg of the amino acid with ethyl isocyanate or KNCO in 20 ml of water and separating the reaction products on Dowex 50 and Dowex 1. The infrared spectrum of the product was identical with that of an authentic sample. *α-Piperidone* was prepared similarly from δ-aminovaleric acid and ethyl isocyanate. Its infrared spectrum was identical with that of a commercial sample after both had been purified further by vacuum sublimation. The infrared spectrum of *α-ketohexamethylenimine*, prepared from ε-aminocaproic acid and ethyl isocyanate, has a single broad peak at 1650 cm^{-1} as its most prominent feature. This peak is most likely a composite of the amide I and amide II bands and is present in the spectra of all three lactams. Several carbamylamino acids have been examined and all show multiple well-resolved bands in this region. The remainder of the spectrum of the 7-membered lactam was consistent with the proposed structure. ε-Aminocaproic acid was formed in 99% yield when the lactam was hydrolyzed in 0.2 M NaOH. *ε-Acetylaminocaproic acid* was formed when ε-aminocaproic acid was treated with 0.75 M KNCO for 3 hours at 25° in 2 M sodium acetate buffer, *p*H 5.3. The reaction products were separated from unreacted starting material and salts on Dowex 50-X8 and then chromatographed on a 2- × 20-cm column of Dowex 1-X8 with 0.4 M acetic acid as eluent. ε-Acetylaminocaproic acid (0.8% yield) emerged at 200 ml and ε-carbamylaminocaproic acid (2.4% yield) at 320 ml. The acetylamino acid gave the expected analyses for C, H, and N, and had an infrared spectrum compatible with the proposed structure. The compound also gave 102% of the expected amount of ε-aminocaproic acid after alkaline hydrolysis and 20% of the expected amount of amino acid after exposure to 6 M HCl at 100° for 15 minutes (in contrast to ε-carbamylaminocaproic which yielded none).

Results

Reaction of Amino Groups with KNCO. In Table I

TABLE I: Apparent Specific Rate Constants for Reactions of Amino Acids and Peptides with KNCO at 30°.

RNH_2	pK_A[a]	[KNCO] (M)	$k_I \times 10^3$[b] (M^{-1} min^{-1})
Tetraglycine	7.75	0.2	275, 279
Triglycine	7.91	0.2	213, 200
Glycylglycine	8.17	0.2	129
		0.4	150[c]
Threonine	9.12	0.2	33.5[d]
		0.4	31.0[c]
Glycine	9.60	0.2	20.2, 20.2
		0.4	21.6[c]
Alanine	9.69	0.2	8.42
		0.8	8.13
		1.0	8.56
β-Alanine	10.19	0.8	5.59
		1.0	5.74
ε-Aminocaproic acid	10.75	1.0	2.04
		2.0	1.98

[a] From Greenstein and Winitz (1961). [b] Calculated from the expression $k_I = -2.3S([H^+] + K_A)/([NCO^-][H^+])$, where S is the slope of a linear plot of log $[RNH_2]_{total}$ versus time. [c] Data from Stark (1965), determinations at *p*H 7.89. All other values were determined at *p*H 8.00. [d] The hydroxyl group of threonine does not react appreciably with KNCO under these conditions.

are shown the apparent specific rate constants for reaction of several amino acids and peptides with KNCO at 30°. The values of k_I (rate = k_I [RNH_3^+][NCO^-]) decrease in a regular fashion and a plot of log k_I versus pK_A is linear, as shown in Figure 1.[2] The best straight line, calculated by the method of least squares, is given by log k_I = 7.94–0.71pK_A. All the determinations of k_I shown in Table I were obtained at cyanate concentrations equal to or greater than 0.2 M. The data of Warner and Stitt (1933) for the ammonium cyanate reaction indicate that the salt effect, large and negative at low ionic strength, is very small above 0.2 M KNCO. It can be seen from the data of Table I that k_I is not influenced significantly by large changes in the concentration of cyanate, i.e., the activity coefficients of the reactants are essentially constant above 0.2 M salt.

Reaction of Alanyllysine with KNCO. It is possible to carbamylate alanyllysine selectively on the α-amino group at *p*H 7 or below, as shown by the results in Table II.[3] Under the particular conditions employed, 85–90% of the product is the α-monocarbamyl derivative. Assuming that the pK_A values for the α- and ε-amino groups of alanyllysine are the same as those for glycylglycine and ε-aminocaproic acid, respectively, it can be calculated from the data of Table I that, after exposure to 0.2 M KNCO for 1 hour at *p*H 7 and 30°, only about 4% of the α-amino groups but 97% of the ε-amino groups should remain unreacted. These calculations are in reasonably good agreement with the experimental findings. If a protein containing 10 residues of lysine were to be selectively modified by cyanate, $(97\%)^{10}$ = 74% of the population of molecules would have no ε-carbamylamino groups and 96% of the population would contain an α-carbamyl group. Therefore, it should be possible to isolate the α-monocarbamyl derivative from such a reaction mixture in good yield. Cole (1961) has reported that insulin, after exposure to urea under conditions that can lead to the formation of cyanate, is carbamylated to the extent of about 14% on both α-amino groups but that the ε-amino group is unmodified.

Relative Rates of Reaction with Ethyl Isocyanate. The relative rates at which glycine, β-alanine, γ-aminobutyric acid, and δ-aminovaleric acid react with ethyl isocyanate in 0.4 M imidazole buffer are shown as a function of pK_A in Figure 2. Details are given under Experi-

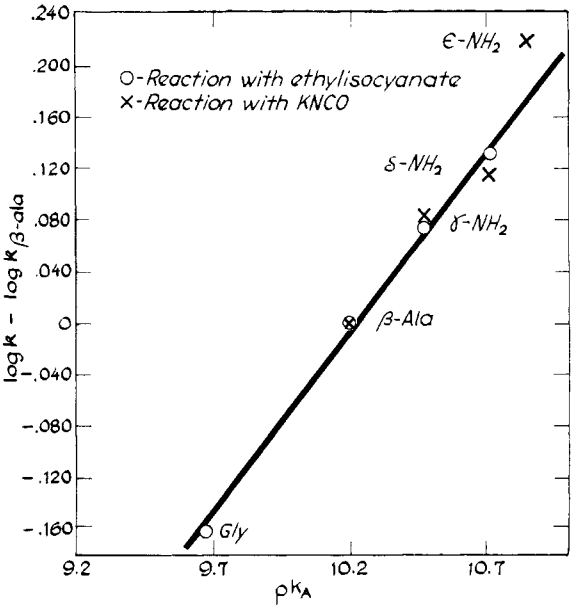


FIGURE 2: Dependence of rate constants upon pK_A for reactions of ω-amino acids with ethyl isocyanate and KNCO at 30°. Relative rates were determined in 0.4 M imidazole buffer as described in the text. For reaction with ethyl isocyanate, average values from duplicate experiments are shown. At the end of the reactions, 92% or more of each amino acid remained unchanged. Reaction with KNCO was done only once. In this case, since 20–40% of each amino acid was modified, the average concentration of each amino acid was used in calculating relative rates.

TABLE II: Products Formed from Reaction of Alanyllysine with KNCO and Subsequent Treatment with Hot 6 M HCl.[a]

	Per Cent of Initial Amount			
Expt	Alanine Hydantoin	Alanine	Homocitrulline	Lysine
1	83	11	5	Not determined
2	92	5	6	88
3	89	8	5	86
Average	88	8	5	87

[a] Reaction conditions: peptide, about 2 × 10^{-3} M; 0.2 M KNCO; *p*H 7.00; 30°; 1 hour. The *p*H was kept constant by automatic addition of dilute acetic acid. Reaction products were heated in HCl, as described in the text.

[2] The reaction can be described equally well by the kinetically equivalent expressions rate = k_m[HNCO][RNH_3] or rate = k_I[NCO^-][RNH_3^+]. Although the first expression reflects the actual mechanism, the second is more convenient to use because above *p*H 5.5 K_{NCO}, the acid dissociation constant of cyanic acid, does not enter into a calculation of the rate since the total concentration of cyanate ion is essentially equal to the molar concentration of KNCO under these conditions. The two rate constants are related by $k_m = k_I K_{NCO}/K_A$, where K_A is the dissociation constant of the protonated amine. It can be shown readily that $(d \log k_I)/(dpK_A) = [(d \log k_m)/(dpK_A)] - 1$, so that the slope of −0.71 shown in Figure 1 corresponds to a slope of +0.29 for the same data plotted according to the molecular mechanism.

[3] In comparing two amino groups, a maximum difference in rate of reaction with cyanate will be obtained at a *p*H more than 1 unit below the lower pK.

mental Procedure. ε-Aminocaproic acid was included in the reaction mixtures also, but gave spurious results because some other reaction product, present even when ε-aminocaproic acid was omitted, chromatographed in the same position on the amino acid analyzer. The pK_A values used in calculating and plotting the data are those of Table III and were determined in 0.5 M NaCl.

TABLE III: Apparent pK_A Values for ω-Amino Acids in 0.5 M NaCl, 25°.[a]

Amino Acid	Apparent pK_A
Glycine	9.67 ± 0.01
β-Alanine	10.20 ± 0.01
γ-Aminobutyric	10.48 ± 0.01
δ-Aminovaleric	10.71 ± 0.07
ε-Aminocaproic	10.85 ± 0.01

[a] The amino acids (0.08 M) were dissolved in CO_2-free 0.5 M NaCl and titrated with standard NaOH. The *p*H values were read on a Metrohm meter using type "U" electrodes; the meter was standardized to *p*H 9.18 ± 0.01 before each titration. The equation $pK_A = pH + \log [RNH_3^+]/[RNH_2]$ was used to calculate pK_A values for several *p*H values near the midpoint of each titration. Each amino acid was titrated at least twice.

The slope of the best straight line through the points for ethyl isocyanate in Figure 2 is 0.28, in excellent agreement with the value 0.29 calculated from Figure 1 for the molecular mechanism with KNCO.

The results of a single experiment with KNCO are also included in Figure 2 but have not been used in determining the slope. Several other experiments with amino compounds and KNCO in the absence of imidazole, not given in detail here, revealed that results in excellent agreement with those of Figure 1 could be obtained with the same techniques used for ethyl isocyanate. It has already been shown that 0.4 M imidazole has no effect on the rates of carbamylation of amines by KNCO (Stark, 1965).

Reaction of Cyanate with Carboxyl Groups. Exposure of γ-aminobutyric acid or δ-aminovaleric acid to KNCO or ethyl isocyanate leads to the formation of 5- and 6-membered lactams in addition to the expected carbamylamino acids. A mixed anhydride of the carboxylic acid and carbamic acid ("carbamylcarboxylate"), formed by addition of a carboxylate anion to HNCO or ethyl isocyanate, is a probable intermediate. Such an anhydride has been shown already to be involved in the formation of several bicyclic compounds from anthranilic acid and phenyl isocyanate (Sheehan and Daves, 1964). In aqueous solution, a readily reversible equilibrium similar to those found for HNCO and sulfhydryl compounds (Stark, 1964) or imidazole (Stark, 1965) would be expected for HNCO and carboxylic acids. In accord with reversibility, the rate of decomposition of KNCO at *p*H 5.2 is unaffected by the presence of 1 M acetate.

Formation of an anhydride activates a carboxyl group and would allow subsequent attack by nucleophiles to result in the formation of amides, esters, and the like. In fact, acetylation of ε-aminocaproic acid was observed in the presence of KNCO and 2 M acetate (see Experimental Procedure). In the case of the γ- and δ-amino acids activation of the carboxyl group allows the lactams to form, since, when these amino acids were exposed to identical conditions in the absence of cyanate, no lactam at all could be detected. The lactams can be separated and identified easily since they are retarded neither by Dowex 50 nor by Dowex 1. Under some experimental conditions, illustrated in Table IV,

TABLE IV: Reaction Products of γ- and δ-Amino Acids and KNCO at 30°.[a]

Product[b]	*p*H	Per Cent of Amino Acid Found as Product[c]: γ-Aminobutyric	Per Cent of Amino Acid Found as Product[c]: δ-Aminovaleric
Lactam	5.30[d]	7.1	6.7
Carbamylamino acid		1.2	0.7
Lactam	5.80[d]	2.5	2.1
Carbamylamino acid		1.2	0.7
Lactam	6.30	1.4	1.2
Carbamylamino acid		2.1	1.3
Lactam	6.80	0.6	0.5
Carbamylamino acid		2.0	1.2

[a] A mixture of all five ω-amino acids, each 0.05 M, was treated with 0.05 M KNCO (*p*H 5.30 and 5.80) or 0.037 M KNCO (*p*H 6.30 and 6.80) for 4 hours at 30°. [b] Carbamyl amino acids were separated from lactams on Dowex 1-X8 as described under Experimental Procedure. [c] The yields of amino acids from hydrolysis of the lactams in 0.2 M NaOH were quantitative and were about 95% from hydrolysis of the carbamyl amino acids. All the values in the table are uncorrected. [d] Reactions at *p*H 5.30 and 5.80 were carried out in 1 M sodium acetate buffer. Therefore "carbamylamino acids" probably include some acetylamino acids as well. See text.

the amount of lactam formed in the presence of KNCO far exceeded the amount of carbamylamino acid. Lactams were not formed from glycine or β-alanine and KNCO under these conditions. A small amount of the

7-membered lactam was isolated from a mixture of ϵ-aminocaproic acid and ethyl isocyanate and was probably present in reaction mixtures with KNCO as well. Peptides would be expected to form by intermolecular reaction of the carbamylcarboxylate of one amino acid with a second molecule of free amino acid. Such reaction products were not detected because they are retained by Dowex 50 unless carbamylation of the free amino group has occurred also. In addition their yield would be low in the relatively dilute solutions of amino acids employed.

The data of Table IV reveal a *p*H dependence for lactam formation that is in reasonably good agreement with the proposed mechanism. If the equilibrium for formation and dissociation of a carbamylcarboxylate is correctly described by the equation

$$RCO_2^- + HNCO + H_2O \rightleftharpoons$$

$$RC(=O)-O-C(=O)-NH_2 + OH^-,$$

a decrease of 1 *p*H unit will increase the concentration of anhydride 100-fold: the forward reaction is ten times faster because there is ten times more HNCO and the reverse reaction is ten times slower because there is ten times less OH^-. Since the concentration of unprotonated amine decreases 10-fold for a decrease of 1 *p*H unit, the net effect predicted is that such a decrease will increase the rate of formation of lactam by 10-fold. In contrast, the rate of carbamylation of the amino group remains unchanged in the *p*H range investigated because the concentrations of RNH_2 and HNCO change by equal amounts in opposite directions.

Discussion

Mechanism of the Reaction of KNCO *with Amines.* The ammonium cyanate–urea conversion, famous as the earliest example of the synthesis of an organic compound from an inorganic precursor (Wöhler, 1828), has been understood well only in recent years. The subject has been reviewed and discussed carefully by Frost and Pearson (1961), who conclude that the alternative mechanisms $NH_3 + HNCO \rightleftharpoons NH_2CONH_2$ and $NH_4^+ + NCO^- \rightleftharpoons NH_2CONH_2$ cannot be distinguished kinetically but that the molecular mechanism is much more likely. The data of Jensen (1959) give particularly strong support to this contention.

A plot of log k_m versus pK_A gives a straight line with slope 0.29 for reaction of the unprotonated amino groups of amino acids and peptides with HNCO in water. This low degree of correlation between nucleophilic reactivity and base strength might be taken to imply that another mechanism is operative in addition to the molecular one, perhaps a mechanism involving the ions. However, a slope of 0.28 is obtained in a plot of log k_m versus pK_A for reaction of amino acids with ethyl isocyanate in water, even though there is no reasonable mechanism involving ions in this case. Therefore, all the present data are in complete accord with the molecular mechanism for the ammonium cyanate–urea conversion and provide some additional evidence for it.

Hall (1964) has shown that data for the reaction of thirty-nine amines with fifteen substrates (electrophiles) can be correlated by the equation of Swain and Scott (1953), $\log k - \log k_{H_2O} = sn$, where k_{H_2O} is the second-order rate constant for hydrolysis of the substrate and s and n are empirical constants for a particular substrate and nucleophile. Values of n can be calculated from rate constants for the amino acids and peptides studied here since k_{H_2O} and s are known for reactions of HNCO. Using these values of n, given in Table V, rate constants can be predicted for reaction of amino acids and peptides with any substrate for which k_{H_2O} and s are known. This approach provides a rational basis for choosing other selective reagents for modifying the amino groups of proteins.

TABLE V: Values of n, a Measure of Nucleophilic Reactivity, for Some Amino Acids and Peptides.

Compound	k_m[a] (M^{-1} sec^{-1})	n[b]
Tetraglycine	74.5	4.53
Triglycine	81.7	4.56
Glycylglycine	98.1	4.61
Threonine	205	4.83
Glycine	400	5.03
Alanine	159	4.76
β-Alanine	418	5.04
ϵ-Aminocaproic acid	530	5.11

[a] Rates of reaction with HNCO in water, from Table I. [b] From the equation $\log k_m - \log k_{H_2O} = sn$. Constants for the hydrolysis of HNCO are $k_{H_2O} = 0.047$ min^{-1} at 18° and $E_a = 19.9$ kcal/mole (Jensen, 1958). From these, $\log k_{H_2O} = 4.79$ at 30°, when k_{H_2O} is expressed in M^{-1} sec^{-1}. The value $s = 1.47$ for HNCO at 30° was interpolated from the values at 18° and 60° given by Hall (1964).

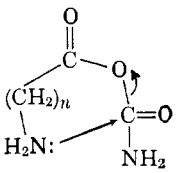
Reaction of Cyanate with Carboxyl Groups. The carbamylcarboxylates studied here are unstable and dissociate readily into carboxylate anion and cyanic acid, with catalysis by hydroxide ion, in a manner very similar to the dissociation of carbamylphosphate dianion (Allen and Jones, 1964), carbamyl mercaptans (Stark, 1964), and carbamylphenols.[4] Carbamylimidazole is unstable also, although it decomposes by a slightly different mechanism that renders the rate independent

[4] D. G. Smyth, manuscript in preparation.

of *p*H above *p*H about 5 (Stark, 1965). Of all the adducts of cyanate with the functional groups of proteins, a carbamylamino group, stable to dilute alkali at room temperature, is the only one that does not decompose readily. The products formed upon reaction of cyanate with serine and threonine will be the subject of a future communication.

It should be borne in mind that treatment of proteins with cyanate in the presence of acetate, particularly at low *p*H, will lead to acetylation of susceptible groups by carbamylacetate and that treatment with cyanate even in the absence of a carboxylate buffer can lead to activation and subsequent reaction of some protein-bound carboxyl groups, although this can be minimized and probably eliminated by carrying out the reaction at a relatively high *p*H.

Arguments against the Direct Transfer of Carbamyl Groups. The carbamylcarboxylates, by analogy with carbamylimidazole, are likely to be very poor carbamylating reagents. This conclusion is indicated also by the observation that glycine and β-alanine react with cyanate at a rate consistent with their pK_a values even at low *p*H, although their carboxyl groups are carbamylated much of the time. Carbamylation of these amino groups apparently does not occur by the mechanism



even though a 5- or 6-membered ring would be an intermediate in the two cases above and a carboxylate anion would be eliminated. In a similar vein, Schreiber and Witkop (1964) have observed that the cyclic intermediate formed by reaction of BrCN with an amino acid reacts with another molecule of amino acid to form a carbamyl peptide rather than a dialkylguanidine.

By a similar argument, carbamylphosphate is probably a very poor carbamylating agent and may function in the cell by providing a safe means of transport for the otherwise reactive cyanate. One then wonders what kind of mechanism is involved in the action of ornithine and aspartate carbamyl transferases, two enzymes that utilize carbamylphosphate as a carbamylating agent. Reichard and Hanshoff (1956) and Reichard (1957) found that ^{32}P-labeled phosphate exchanged with carbamylphosphate only in the presence of both amino acid and enzyme and that ^{14}C-labeled amino acid exchanged with carbamylamino acid only in the presence of both phosphate and enzyme. These observations were taken as evidence that the reaction proceeds by a single displacement mechanism. If a single displacement is in fact the mechanism, the carbamyl transferases must activate the unreactive carbonyl carbon of carbamylphosphate by increasing its electrophilic character. Another possibility not inconsistent with the data is that an enzyme-mediated dissociation of carbamylphosphate occurs only when the amino acid is also bound to the enzyme and that the cyanic acid produced is the real carbamylating agent.

Although both carbamyl transferases are inhibited by —SH reagents (Reichard, 1957; Reichard and Hanshoff, 1956; other references in Cohen and Marshall, 1962), it is very unlikely that *S*-carbamyl proteins are intermediates in the enzymatic reactions, both because the results of Reichard are inconsistent with the formation of a carbamyl enzyme and because *S*-carbamyl compounds are not carbamylating agents either: *S*-Carbamylcysteine decomposes at about the same rate as other *S*-carbamyl compounds and yields cysteine and cyanic acid as products rather than *N*-carbamylcysteine (Stark, 1964). If direct *S*- to *N*-carbamyl transfer could occur, it would be facilitated greatly in this case by formation of an intermediate 5-membered ring.

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