Reactions of Cyanate with Functional Groups of Proteins. IV. Inertness of Aliphatic Hydroxyl Groups. Formation of Carbamyl- and Acylhydantoins*

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ABSTRACT: The hydroxyl groups of serine, threonine, dipeptides of serine, ethanol, and glycolic acid do not react with cyanate in aqueous solution to a detectable extent under conditions that are extreme for modification of other functional groups in proteins. Carbamyl- and, to a lesser extent, acetyl-α-amino acids (models for the carboxyl termini of peptides and proteins) do react with cyanate at pH values below 7 to form N-substituted hydantoins, probably by a mechanism involving mixed anhydrides of the carboxylic acid with carbamic and isocyanic acids. The compounds 1-carbamyl-5-methyhydantoin and 1-carbamyl-5-hydroxymethylhydantoin were prepared by reaction of alanine and serine with KNCO, and 1-acetyl-5-methylhydantoin was prepared similarly from acetylalanine. These N-substituted hydantoins have pK_A values near 7 and the ionized forms absorb maximally near 225 mμ. The carbamylhydantoins are stable in alkali, but the acetyl compound is deacetylated rapidly in dilute sodium hydroxide.

Cyanate[1] in aqueous solution has been suggested as a reagent for the selective modification of amino groups in proteins (Stark, 1965b). Although carbamylation of the side chains of cysteine (Stark, 1964), histidine (Stark, 1965a), and tyrosine residues (D. G. Smyth, unpublished observations) may occur when a protein is treated with cyanate in aqueous solution, the reactions are reversible under mild conditions and the original functional groups can be regenerated readily. In contrast, carbamylamines are quite stable. Cyanate reacts with carboxyl groups also, but appreciable reaction does not occur above pH 7. The carbamylcarboxylates that are formed dissociate again readily, but are reactive and may give rise to a variety of products, as shown in this paper and by Stark (1965b).

The present report is concerned in large part with whether aliphatic hydroxyl groups react with cyanate under conditions that are useful for carbamylating amino groups. Aliphatic hydroxyl groups do react with isocyanic acid, generated *in situ* from sodium or potassium cyanate and a strong acid, to yield either urethans or allophanates, but good yields are obtained only in anhydrous media (Werner and Gray, 1947; Loev and Kormendy, 1963). When Skinner *et al.* (1956) attempted to obtain an *O*-carbamyl derivative from N-carbobenzyloxyserine and aqueous KNCO, only starting material was recovered. The data presented now show that the aliphatic hydroxyl groups of proteins would not be expected to react with aqueous cyanate to a significant extent under conditions that suffice for complete reaction of amino groups in proteins. In view of this, the addition of cyanate to a *reactive* serine residue in several proteolytic enzymes under relatively mild conditions (Shaw *et al.*, 1964) is especially unusual.

A detailed examination of the reaction products formed from cyanate and serine revealed, in addition to the expected *N*-carbamylserine, a new compound that still retains an hydroxyl group but no longer has a carboxyl. This compound, 1-carbamyl-5-hydroxymethylhydantoin, seems to be formed by interaction of the carbamylcarboxylate with isocyanate anion, followed by cyclization of the resulting mixed anhydride of the carboxylic acid and isocyanic acid. Cyanate can be used to prepare analogous acylhydantoins from *N*-acetylamino acids and from peptides.

Results

Reactivity of Aliphatic Hydroxyl Groups toward Cyanate in Aqueous Solution. Carbamylation occurred only at the amino group when serylglycine, glycylserine, and serylleucine were treated exhaustively with concentrated KNCO at neutral or slightly alkaline pH. In each case, only one peak that yielded an amino acid after hydrolysis was obtained when the reaction mixture was chromatographed on Dowex 50 in water or dilute acetic acid. These products were identified unambiguously as *N*-monocarbamylpeptides by elemental analysis. Serine and threonine both gave the

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[1] The term "cyanate" is used in a generic sense throughout the text and is intended only to indicate that the reagent is a salt of cyanic (or isocyanic) acid and not that the species NCO^- participates in the reaction as such. Reaction of "cyanate" with amines, mercaptans, carboxylate anions and the like undoubtedly involves nucleophilic attack on the species HNCO. Where the species NCO^- *is* a reactant, the term "isocyanate anion" is used.

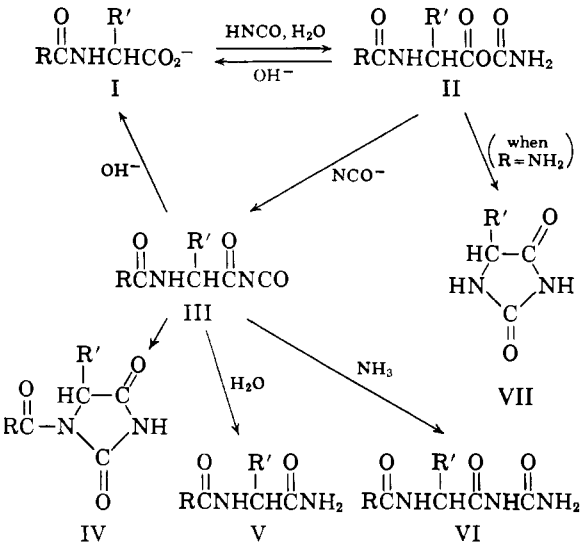


FIGURE 1: Interactions of carbamyl- or acylamino acids with cyanate.

corresponding carbamylamino acids, carbamylhydantoins, and hydantoins when treated with excess cyanate at acidic pH values, but the hydroxyl groups remained unmodified.

The reaction of cyanate with ethanol was studied in another type of experiment. Since carbamylation of the hydroxyl group would occur according to the equation

$$EtOH + NCO^- + H_2O \rightleftharpoons EtO\overset{\overset{O}{\|}}{C}NH_2 + OH^-$$

the rate at which protons must be added to keep the pH constant is a measure of the rate of carbamylation. However, hydrolysis and polymerization of cyanate also result in the liberation of hydroxide ions and must be corrected for. The uptake of protons at pH 7 and 30° by a solution of KNCO, 0.5 M in 50% aqueous ethanol, was measured with a pH-Stat. The rate at which acid was added in this experiment was somewhat less than the rates of blank determinations in which similar mixtures of tetrahydrofuran or acetone and water were used, indicating that there is little or no carbamylation of the aliphatic hydroxyl group. The rates were much faster in all three of the organic solvent–water mixtures than in water alone, probably because the pK_A of cyanic acid increases with decreasing dielectric constant, resulting in more rapid hydrolysis and polymerization of the cyanate.

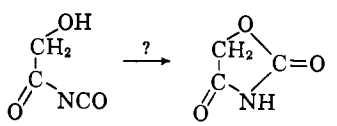
In order to determine whether or not carbamates are stable enough to be detected if formed, the rate of addition of alkali necessary to stabilize the pH of 5 M urethan at 12.5 was determined at 30°. In this case, either dissociation to ethanol and cyanate or hydrolysis would require the addition of alkali. The rate was not significantly higher than that of a control without urethan.

Reaction of Carbamyl- and Acylamino Acids with Cyanate. The variety of products that could be formed was investigated by using several derivatives of amino acids as starting materials. The reactions are summarized in Figure 1.

Mixtures of serine with excess cyanate were allowed to stand overnight and then acidified. When excess acetic acid was used, only the carbamylamino acid (I) and *N*-carbamylserine hydantoin (IV) were found, but, when the reaction mixture was acidified to pH 1–2 with HCl, a substantial amount of serine hydantoin (VII) was present as well. (Hydantoins, pK_A about 9, can be separated readily from carbamyl- or acylhydantoins, pK_A about 7, on short columns of Dowex 1-X8 in 0.2 M potassium phosphate buffer, pH 6.5.) No hydantoin was formed in control experiments with *N*-carbamylserine and HCl in the absence of cyanate. Therefore the hydantoin probably arises by cyclization of the carbamylcarboxylate (II). Because of the facile formation of the hydantoin and carbamylhydantoin, it is difficult to synthesize pure *N*-carbamylserine from serine and cyanate unless a slight excess of the amino acid is used and acidification is avoided. Qualitatively similar results were obtained with threonine. When carbamylalanine was the starting material, a 50% yield of the corresponding carbamylhydantoin (IV) was obtained in duplicate analytical scale experiments carried out under the preparative conditions given in the Experimental Section.

Procedure

The interaction of glycolic acid with cyanate was examined to determine whether or not a favorably situated hydroxyl group could react intramolecularly with the carboxylic–isocyanic anhydride to form an



oxazolidinedione. However, only starting material and decomposition products of cyanate were isolated from Dowex 1 and Dowex 50 following treatment of 3.8 g of glycolic acid with KNCO, initially 3 M, for 2 hr at pH 6 and 35°, indicating either that a substantial amount of oxazolidinedione does not form or, less likely, that it is unstable. This finding concurs with the observation that no derivative with a modified hydroxyl group was isolated from reaction mixtures of cyanate and serine.

When acetylalanine was treated with 2.3 M KNCO and the reaction mixture acidified subsequently with excess acetic acid, the compounds not retarded by Dowex 1-X8 (*i.e.*, that do not have an anionic group) and that yield alanine after alkaline hydrolysis accounted for 6% of the starting material. In another experiment, the yield was increased to 12% in 6 M KNCO; the pH was kept at about 5 with HCl in this case. Chromatography on Dowex 50-X8 (see Experimental Section) revealed that about 25% of the mixture

of products is not the acetylhydantoin but probably the amide (V) and acylurea (VI) formed by reaction of water or NH_3 with the intermediate carboxylic–isocyanic anhydride (III). However, these products were not characterized. The lower yield of N-substituted hydantoin obtained with an acylamino acid indicates that the α-NH- group does not react as readily when acylated as when carbamylated, giving the intermediate III a greater opportunity to react intermolecularly.

The particular procedure given in the Experimental Section for synthesis of acetylalanine hydantoin from acetylalanine demonstrates that this product is formed by reaction with cyanate alone, in support of the reaction mechanism shown in Figure 1. However, a much better yield is obtained if cyanate is added after the carboxyl group has been activated already, for example, with acetic anhydride. This reaction is now being investigated in detail with peptides.

Discussion

The mechanisms given in Figure 1 are supported both by the evidence in this paper and by observations of other workers. Although very little work has been done in aqueous systems, acyl isocyanates are known (for example, acetyl isocyanate has been synthesized from acetyl chloride and AgNCO (Belliter, 1903)), and the reactions of such compounds with water to give amides (with elimination of CO_2) and with amines to give acylureas have been described (Johnson and Bublitz, 1957; Walling *et al.*, 1963). The ability of isocyanate anion to function as a nucleophile has been documented also. For example, in aqueous solution, the well-known polymerization of cyanate to the cyclic trimer cyanuric acid must involve a nucleophilic attack of NCO^- on HNCO; in accord with such a mechanism, we have observed that the rate of this reaction decreases rapidly as the pH is raised. In organic solvents, isocyanate anion is even capable of adding to the carbonyl groups of aldehydes and ketones (Hoover *et al.*, 1963).

We have not investigated the interaction of cyanate with guanidinium and indole groups because the possibility of appreciable reaction seems remote. The only other potentially reactive groups in proteins that have not been examined are amides. From the work of Werner and Gray (1947), who found that allophanates were not formed from interaction of HNCO and alcohols in the presence of water even under conditions where urethans were formed, it seems highly unlikely that amides are reactive under conditions useful for modifying other groups in proteins.

Experimental Section

Materials and Methods. Reagent grade KNCO was recrystallized from ethanol–water at a maximum temperature of 50°. Peptides and amino acids from various commercial sources were homogeneous on the amino acid analyzer (Spackman *et al.*, 1958). Other compounds melted sharply within a few degrees of literature values.

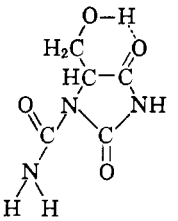


FIGURE 2: 1-Carbamyl-5-hydroxymethylhydantoin.

Infrared and ultraviolet spectra were obtained with Perkin-Elmer Model 237 and Cary Model 14 spectrophotometers and proton magnetic resonance spectra with a Varian HR-100 spectrometer. Melting points were taken with a Fisher-Johns block and are uncorrected.

Synthesis of 1-Carbamyl-5-methylhydantoin (IV, R = NH_2; R′ = CH_3). One gram of DL-alanine and 7.5 g of KNCO were dissolved in 20 ml of water. After 4 hr at room temperature, 30 ml of 50% acetic acid was added and, after 1 hr more, the solution was taken to dryness on a rotary evaporator. The residue was suspended in 60 ml of water and the soluble portion was desalted on a 2 × 40 cm column of Dowex 50-X8, hydrogen form, in water. The first 460 ml of effluent was concentrated to about 10 ml and the soluble portion was passed through a 2 × 20 cm column of Dowex 1-X8, acetate form, in 0.05 M acetic acid. The first 150 ml of effluent was taken to dryness and the residue was chromatographed on a 2 × 40 cm column of Dowex 50-X8, hydrogen form, in water. The only peak that yielded alanine after alkaline hydrolysis emerged between 140 and 170 effluent ml (yield, 130 mg; mp 192–202°). After two recrystallizations from water, 45 mg of material were obtained, mp 223–225°.

Anal. Calcd for $C_5H_7N_3O_3$: C, 38.22; H, 4.47; N, 26.74. Found: C, 38.01; H, 4.40; N, 26.43.

An ultraviolet spectrum in 0.1 M NaOH revealed a single peak, λ_{max} 225 mμ (ϵ_m 17,000). The compound was stable in this solvent, since the absorbancy had not changed after several hours. The pK_A', determined spectrophotometrically in 0.033 M phosphate buffers, was 7.0. The compound gave 98% of the theoretical amount of alanine after hydrolysis for 16 hr at 110° in 0.2 M NaOH.

1-Carbamyl-5-hydroxymethylhydantoin (IV, R = NH_2; R′ = CH_2OH) was prepared from 500 mg of L-serine and 1 g of KNCO by a procedure analogous to that used for 1-carbamyl-5-methylhydantoin, but without the step in which Dowex 1 was used. Only two ninhydrin-positive peaks that yielded serine after alkaline hydrolysis were obtained upon chromatography on Dowex 50-X8. These were identified as *N*-carbamylserine (65%) and the carbamylhydantoin (18%). Similar results were obtained with threonine and cyanate. After one recrystallization from water, carbamylserine hydantoin melted at 193–195°, with decomposition.

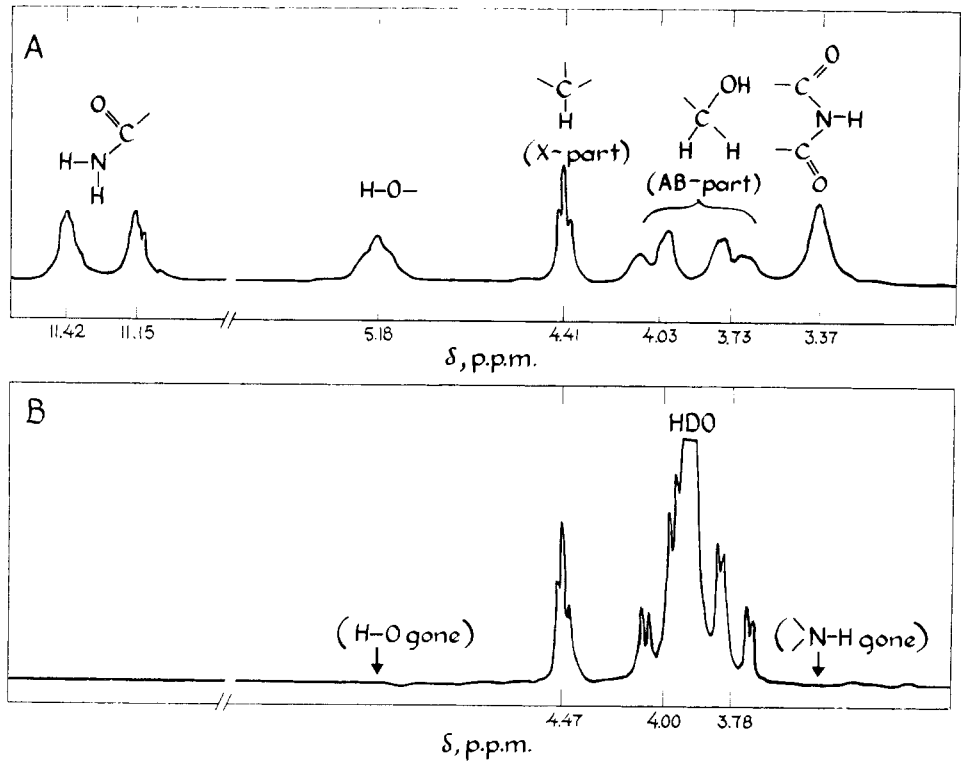


FIGURE 3: Proton magnetic resonance spectra of 1-carbamyl-5-hydroxymethylhydantoin at 100 Mc. A, in deuterated dimethyl sulfoxide, with tetramethylsilane as a reference; B, the same after addition of D_2O.

Anal. Calcd for $C_5H_7N_3O_4$: C, 34.68; H, 4.08; N, 24.27. Found: C, 34.70; H, 4.05; N, 24.30.

The ultraviolet spectrum in 0.05 M NaOH revealed a single peak, λ_{max} 226 mμ (ϵ_m 15,500). The pK_A', determined spectrophotometrically in 0.033 M phosphate buffer, was 6.9. No other ionization was observed titrimetrically, indicating that a COOH group is absent. The compound gave a 26.5% recovery of serine after hydrolysis in 0.2 M NaOH at 110° for 18 hr.

These facts are consistent either with the carbamylhydantoin or with the isomeric 5-ureido-1,3-tetrahydrooxazine-2,4-dione, a compound that would be formed if the OH rather than the NH were to participate in the cyclization of III (R = NH_2; R′ = CH_2OH). An attempt was made to differentiate these possibilities with a mass spectrometer but the compound decomposed, even with a direct inlet system. The infrared spectrum (KBr pellet) revealed the three carbonyl bands expected of either compound (at 1690, 1730, and 1780 cm^{-1}; serine hydantoin lacks the band at 1690 cm^{-1}) but failed to show a strong band in the region of 1000 cm^{-1} for OH deformation. A medium to weak band at about 940 cm^{-1}, present in the spectrum of both serine hydantoin and carbamylserine hydantoin, was absent in the spectrum of carbamylalanine hydantoin. However, assignment of this band to a hydrogen-bonded group is not completely convincing because the spectra are very complex. Similarly, interpretation of differences in the region of 3500 cm^{-1} (due to OH stretching) is complicated by multiple bands in the same region due to NH groups.

The structure of carbamylserine-hydantoin, shown in Figure 2, was proved unambiguously by proton magnetic resonance spectroscopy. In the spectrum shown in Figure 3A, the peak at 5.18 ppm, due to the OH proton, is broadened considerably by spin–spin coupling with the CH_2 protons. A critical point of evidence in proving that this peak is due to OH in the grouping CH_2OH (present only in the carbamylhydantoin) and not NH in the grouping $-\overset{|}{C}HNH\overset{|}{C}=O$ (present only in the oxazinedione) is that the peaks due to CH_2 protons are broadened reciprocally by coupling with the OH proton. When D_2O is added, so that all peaks due to exchangeable protons are lost, the CH_2 bands are sharpened considerably (Figure 3B). The spectrum indicates that the OH group is hydrogen bonded intramolecularly because the CH_2 protons are not equivalent. They form the AB part of a typical ABX spectrum (Roberts, 1961) and the expected 8 peaks are shown to be resolved clearly in Figure 3B. The >CH– group (X part) gives the expected triplet. The bands at 11.15 and 11.42 ppm in Figure 3A are due to nonequivalent NH_2 protons.

1-Acetyl-5-methylhydantoin (IV, R and R′ = CH_3) was prepared from 2 g of acetyl-DL-alanine and 10 g

of KNCO in 30 ml of 33% acetic acid. After 1 hr at room temperature, the reaction mixture was acidified to pH 1 with HCl and taken to dryness. The organic products were extracted with hot absolute ethanol, then separated from the bulk of unreacted starting material on Dowex 1 after removal of the solvent. Three peaks that yielded alanine after alkaline hydrolysis were separated on a 2 × 95 cm column of Dowex 50-X8 in water. Peak I (300 ml) was acetyl-DL-alanine, peak II (390 ml) was the crude acetylhydantoin (290 mg), and peak III (540 ml) was probably acetylalanineamide and acetylalanylurea (V and VI, R and R′ = CH_3). Peak II was rechromatographed on Dowex 1-X8 (acetate form in 0.05 M acetic acid) and again on Dowex 50. Crystalline material (48 mg, mp 123–125°) was obtained when the peak tubes were evaporated to dryness.

Anal. Calcd for $C_6H_8N_2O_3$: C, 46.15; H, 5.16; N, 17.94. Found: C, 46.00; H, 5.21; N, 17.99.

The ultraviolet spectrum in 0.05 M NaOH revealed a peak at 226 mμ (ϵ_M 18,900). The compound decomposed rapidly under these conditions (half-time 6.4 min at 30°) to give a product whose ultraviolet spectrum and extinction coefficient were indistinguishable from those of authentic alanine hydantoin. Consequently, ϵ_M for the acetylhydantoin was obtained by extrapolation. The pK_A, determined spectrophotometrically in 0.033 M phosphate, was 7.2. The compound gave 102% of the theoretical amount of alanine after hydrolysis for 16 hr at 110° in 0.5 M NaOH.

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