New Amino Acids Derived from Reactions of ε-Amino Groups in Proteins with α,β-Unsaturated Compounds*

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ABSTRACT: Reduced bovine serum albumin, reduced whole wheat gluten, and polylysine were reacted with acrylonitrile and with methyl acrylate. Amino acid analyses of hydrolysates of the modified proteins revealed that the formation of two new compounds was accompanied by a decrease in lysine. Evidence indicates that the new amino acids are ε-*N*,*N*-bis(β-carboxyethyl)-L-lysine and ε-*N*-(β-carboxyethyl)-L-lysine. Kinetic studies on the hydrolysis of ε-*N*,*N*-bis(β-cyanoethyl)-L-lysine under both acidic and basic conditions indicate that this amino acid undergoes decyanoethylation and its hydrolysis product undergoes decarboxyethylation.

Amino acid analysis of hydrolysates of modified proteins does not always reveal the ratio of mono- to disubstituted derivatives actually formed during the reaction of ε-amino groups of lysine side chains with α,β-unsaturated compounds. The original ratio may be altered during hydrolysis in favor of the monosubstituted derivative.

Following the demonstration that acrylonitrile can be used as a specific blocking agent for sulfhydryl groups in milk proteins (Weil and Seibles, 1961; Cavins and Friedman, 1967), several groups of investigators showed that other functional groups in proteins, especially ε-amino groups of lysine side chains, also react with acrylonitrile under certain conditions (Friedman and Wall, 1964; Friedman, 1967; Kalan *et al.*, 1965; Riehm and Scheraga, 1966). In a previous communication it has been shown that SH groups in aminothiol acids react about 300 times faster with acrylonitrile than amino groups at comparable basicities and steric environments (Friedman *et al.*, 1965).

The present studies were intended to characterize new amino acids formed during alkylation of sulfhydryl groups in reduced whole wheat gluten and reduced bovine serum albumin with acrylonitrile or methyl acrylate.

The reaction of the ε-amino group in α-*N*-CBZ-L-lysine[1] with two related reagents (acrylonitrile and methyl acrylate) will be considered with reference to the indicated transformations.

Experimental Section

Materials. Whole gluten was prepared from Ponca wheat (Jones *et al.*, 1961); bovine serum albumin (four-times crystallized) was obtained from Pentex;[2] polylysine hydrogen bromide, DP 300, from Pilot; L-lysine, free base, from Sigma; α-*N*-CBZ-L-lysine from Cyclo; acrylonitrile from Matheson; methyl acrylate from Rohm & Haas; and mercaptoethanol from Eastman. All liquid reagents were redistilled before use.

Amino Acid Analyses. These were carried out on a Phoenix K-8000 VG amino acid analyzer utilizing both the Piez–Morris (1960) and Spackman–Stein–Moore (1958) systems.

Modification of Proteins. Reduction and alkylation of whole wheat gluten and bovine serum albumin were carried out as follows. To 0.5 g of the protein in 20 ml of 6 M urea (pH 8.0)–Tris buffer (Benesch *et al.*, 1955) saturated with nitrogen was added mercaptoethanol in an 80:1 ratio to the number of disulfide bonds. The solution was stirred for 16 hr at room temperature and then a 2:1 molar ratio of either acrylonitrile or methyl acrylate to mercaptoethanol was added. Stirring was continued for 4 more hr; the reaction mixture was acidified with acetic acid, dialyzed against 0.01 N acetic acid, and lyophilized. Modified and native protein samples were hydrolyzed with constant-boiling HCl at 110° for 24 hr in sealed tubes. The ratio of acid to protein was 1 ml to 2 mg.

The complete modification of ε-amino groups in polylysine was carried out as follows. To a solution of 209 mg of polylysine hydrogen bromide (1 mmole of lysyl residues) in 25 ml of water was added 1 ml of either acrylonitrile (15 mmoles) or methyl acrylate (11 mmoles) with vigorous stirring under an atmosphere of nitrogen

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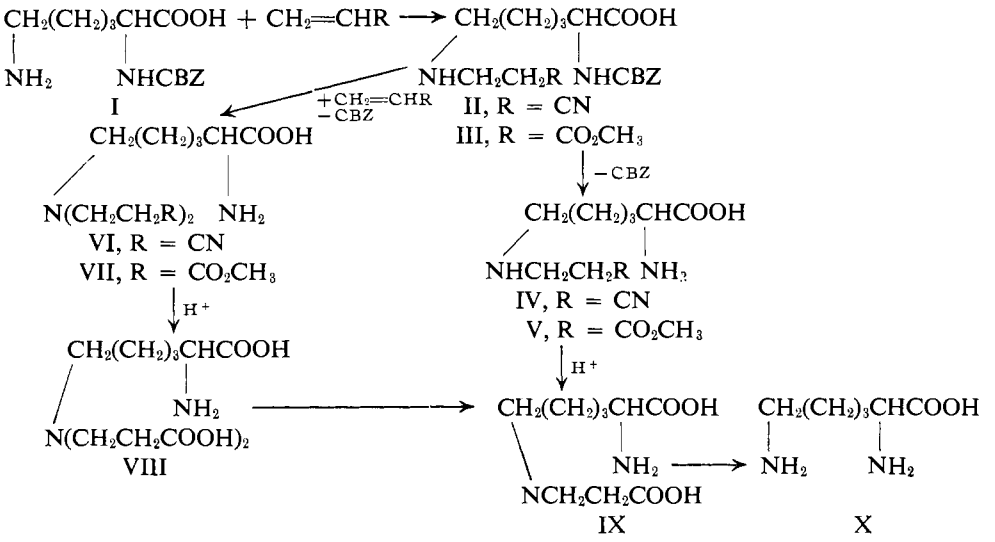
[1] Abbreviation used: α-*N*-CBZ-L-lysine, α-*N*-carbenzoxy-L-lysine.

[2] Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

SCHEME I



and 0.1-ml aliquots of 0.2 M LiOH until the reaction mixture was pH 8. This pH was maintained for 10 hr with LiOH solution. The mixture became cloudy as the reaction progressed, and after 1 hr a precipitate formed. The reaction mixture was then dialyzed against water and evaporated to dryness. A sample was hydrolyzed by refluxing in 100 ml of 6 N HCl for 24 hr and analyzed on an amino acid analyzer.

ε-N,N-Bis(β-cyanoethyl)-L-lysine (VI). This new amino acid was prepared as follows. *α-N*-CBZ-L-lysine (I) (560 mg, 2 mmoles) was suspended in 20 ml of water at 30°. To the suspension was added 0.2 M LiOH until the pH reached 10, at which point the lysine derivative was in solution. Three aliquots of acrylonitrile (2 mmoles) were added at 30-min intervals and the pH of the reaction mixture was maintained at 10 by addition of LiOH solution. The reaction was complete in 90 min. The mixture was then evaporated to dryness under reduced pressure, taken up in water, and neutralized with HCl. The oily layer that came out of solution on neutralization was extracted with chloroform, the chloroform was evaporated, and the residue was taken up in methanol. The methanol solution was hydrogenated in the presence of 100 mg of 10% palladium on charcoal for 30 min to remove the carbobenzoxy group (Greenstein and Winitz, 1961). The compound was obtained as white needles after three crystallizations from hot ethanol containing a trace of water: yield, 355 mg (71.0%); mp 198–200° not corrected.

Anal. Calcd for $C_{12}H_{20}N_4O_2$ (252): C, 57.15; H, 7.94; N, 22.22. Found: C, 57.06; H, 8.19; N, 22.50. Calcd for nitrile nitrogen: 11.1. Found: 10.9.

The infrared spectrum taken as a KBr pellet contained a strong peak at 2245 cm^{-1}, which is characteristic of nitrile groups.

Hydrolyses of VI. Studies on the hydrolysis of *ε-N,N*-bis(β-cyanoethyl)-L-lysine (VI) were made in various concentrations of acids and bases in sealed tubes at 110°. Basic hydrolysates were neutralized with HCl. Each hydrolysate was evaporated to dryness and the residue was dissolved in H_2O. This process was repeated several times. Appropriate aliquots were then taken for amino acid analyses.

Extended Hydrolysis and Chromatography of ε-N,N-Bis(β-cyanoethyl)-L-lysine (VI). Compound VI (50 mg) was heated at 110° in a sealed tube for 48 days in 10 ml of 6 N HCl. The dry reaction product was dissolved in 10 ml of 1 N HCl and applied to a column (60 × 4 cm) of Dowex 50 W-X8 (200–400 mesh) which had been equilibrated with 1 N HCl. The compounds were eluted with increasing HCl concentrations (Christianson *et al.*, 1960).

Results

Disulfide bonds in bovine serum albumin and whole wheat gluten were reduced with mercaptoethanol, and the generated sulfhydryl groups were reacted either with acrylonitrile or methyl acrylate. Hydrolysates of the modified proteins contained two new amino acids in addition to *S*-carboxyethylcysteine. Appearance of the new compounds was accompanied by a decrease in lysine content while other amino acids remained essentially unchanged. The loss of lysine was 49.6% for wheat gluten and 51.9% for bovine serum albumin. The new amino acids eluted at 352 and 425 ml on the Piez–Morris (1960) system (Table I). Hydrolysates of polylysine, in which the ε-amino groups were completely modified either with acrylonitrile or methyl acrylate, contained only these new amino acids.

The new amino acid *ε-N,N*-bis(β-cyanoethyl)-L-lysine (VI) was synthesized from *α-N*-CBZ-lysine (I) and acrylonitrile. Hydrolysis of the nitrile groups under conditions used for protein hydrolysis gave two ninhydrin-positive compounds whose elution positions correspond to the new amino acids found in hydrolysates of alkylated proteins.

The hydrolysis of the dicyanoethyl derivative VI

TABLE I: Elution Position (milliliters) of New Amino Acids and Standards.

Amino Acid	Piez–Morris (1960) System	Spackman–Stein–Moore (1958) System	
		15-cm Column	150-cm Column
ε-*N*-(β-Cyanoethyl)-L-lysine (IV)	514	63	
ε-*N*,*N*-Bis(β-cyanoethyl)-L-lysine (VI)	435	22	790
ε-*N*-(β-Carboxyethyl)-L-lysine (IX)	425	~20	538
ε-*N*,*N*-Bis(β-carboxyethyl-L-lysine (VIII)	352	~20	344
L-Lysine	512	55	
L-Phenylalanine	416	~20	441
L-Methionine	335	~20	345

was investigated as a function of time (Table II) and as a function of the reaction medium (Table III). The basis for the structural assignments of compounds in the tables is given in the Discussion section.

The elution positions on the HCl eluted Dowex 50 column (Christianson *et al.*, 1960) of the two new amino acids derived from ε-*N*,*N*-bis(β-cyanoethyl)-L-lysine, presumed to be VIII and IX, are shown in Figure 1 (peaks 2 and 3, respectively).

The reaction product of α-*N*-CBZ-L-lysine (I) treated with 2 equiv of acrylonitrile at 0° for 2 hr at pH 10 followed by hydrogenation consisted of three ninhydrin-positive compounds in addition to a trace of ammonia present in the starting material (Figure 2). This reaction produced a new compound, presumably the monocyanoethyl derivative IV, which elutes at 63 ml on the 15-cm Spackman *et al.* (1958) column (Table I). Hydrolysis of the reaction mixture in 6 N HCl for 24 hr at 110° resulted in loss of ε-*N*-monocyanoethyllysine and the appearance of ε-*N*-monocarboxyethyllysine. In an effort to prepare the monocyanoethyl derivative IV, α-*N*-CBZ-lysine (I) was treated with 1 equiv of acrylonitrile at 0°. The final reaction product was a mixture of L-lysine and IV when the reaction was carried out for 2 hr. However, when the reaction period was increased beyond this time, the concentration of the monocyanoethyl derivative started decreasing with a concomitant increase in that of the dicyanoethyl derivative. Additional unsuccessful reactions were carried out in which the pH of the medium, time of reaction, and concentration of reactants were varied.

Attempts to separate lysine from IV on a preparative scale were unsuccessful. Paper chromatography with the lower phase of butanol–acetic acid–water (25:6:25) gave a slight separation. Chromatography on a cellulose column with the same solvent system gave no separation. Similarly, preparative chromatography on Dowex-50, Sephadex G-10, Bio-Gel P-2, silica gel, and alumina columns did not separate these two amino acids.

TABLE II: Effect of Time on Hydrolysis of ε-*N*,*N*-Bis(β-cyanoethyl)-L-lysine (VI).

Hydrolysis Time (hr)	Peak Area of VIII[a]	Peak Area of IX	Ammonia (μmoles)/250 μg	Peak Area Ratios of VIII:IX
6	16.45	0.77	2.0	21.4
12	16.66	0.84	1.99	19.8
24	17.66	1.33	2.19	13.2
48	15.13	1.80	1.96	8.4
336	7.00	10.46	2.00	0.67

[a] Peak areas were determined from chromatograms obtained on an amino acid analyzer with the Piez–Morris (1960) system.

TABLE III: Effect of Media on Hydrolysis (24 hr, 110°) of ε-*N*,*N*-Bis(β-cyanoethyl)-L-lysine (VI).

Reaction Medium (N)	% of Total[a]					
	IV	VI	VIII	IX	X	Unknown
NaOH						
6.0			6	67	26	
3.0			7	64	29	
1.0			4	76	20	
0.1				60	40	
0.01	28	2		3	37	30
H_2O	24	7			36	33
HCl						
0.01	24	10			66	
0.1		43			57	
1.0		16	47	18	19	
3.0			93	7		
6.0			95	5		
12.0			100			

[a] Peak areas calculated by $H \times W$ method.

Discussion

In principle, cyanoethylation of ε-NH_2 groups of lysine side chains in proteins could produce ε-*N*-mono- and ε-*N*,*N*-dicyanoethyl derivatives. Hydrolysis of the modified proteins with HCl should also hydrolyze the nitriles to carboxy groups. It was therefore assumed that the new amino acids which appeared in the modified protein hydrolysates were ε-*N*,*N*-bis(β-carboxy-

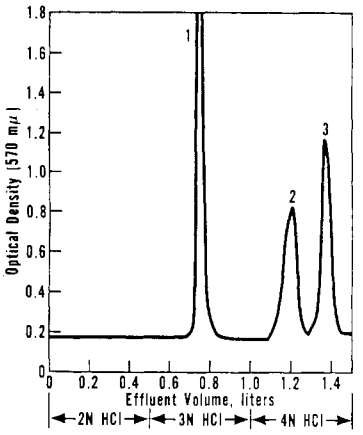


FIGURE 1: Dowex 50 chromatogram of 48-day hydrolysate (6 N HCl, 110°) of ε-*N*,*N*-bis(β-cyanoethyl)-L-lysine (VI): (1) ammonia, (2) ε-*N*,*N*-bis(β-carboxyethyl)-L-lysine (VIII), and (3) ε-*N*-(β-carboxyethyl)-L-lysine (IX).

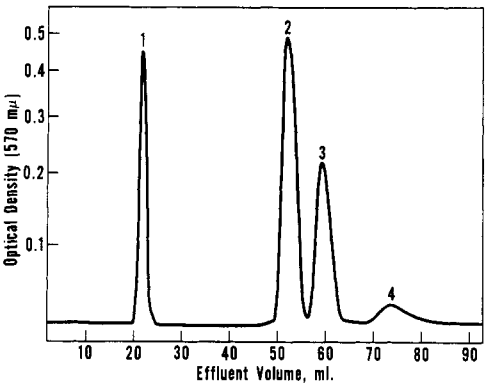


FIGURE 2: Elution positions of (1) ε-*N*,*N*-bis(β-cyanoethyl)-L-lysine (VI), (2) L-lysine, (3) ε-*N*-(β-cyanoethyl)-L-lysine (IV), and (4) ammonia on the 15-cm column (Spackman *et al.*, 1958).

ethyl-L-lysine (VIII) and ε-*N*-(β-carboxyethyl)-L-lysine (IX). The synthesis of these amino acids was undertaken so that elution positions could be compared.

A chromatographically pure sample of the new amino acid ε-*N*,*N*-bis(β-cyanoethyl)-L-lysine (VI) was synthesized. It was suspected that VI was one of the compounds originally formed in the protein which would yield the dicarboxyethyl derivative VIII on hydrolysis of the nitrile groups. Surprisingly, hydrolysis of the nitrile groups under conditions used for protein hydrolysis gave not one but two ninhydrin-positive compounds other than ammonia. Their elution positions were identical with those of the new amino acids found in hydrolysates of the reduced and alkylated proteins described previously.

The kinetics of the degradation of the dicyanoethyl derivative VI was then investigated. Hydrolysis for less than 6 hr produces essentially one compound other than the expected two equivalents of ammonia from the nitrile groups (Table II). On the basis of this observation it is concluded that this compound is the dicarboxyethyl derivative VIII. When the time of hydrolysis is extended, the appearance of a new compound was noted with a concomitant decrease in VIII. This conversion did not produce additional ammonia (Table II). On the basis of all the experimental evidence it is concluded that this reaction involved a decarboxyethylation of the dicarboxyethyl derivative VIII to the monocarboxyethyl derivative IX.

The results in Table III demonstrate that the cyanoethylated proteins are subject to six different reactions under the conditions used for their hydrolysis. These are loss of one or both cyanoethyl side chains (alkaline or neutral pH), hydrolysis of one or both cyano groups (acid or basic pH), and loss of one (acid pH) or both (alkaline pH) carboxyethyl side chains. The extent of these consecutive and competitive reactions is governed by the acidity of the medium and by time of the reaction, as indicated in Table III. These observations show that amino acid analysis of hydrolysates of modified proteins may not provide the ratio of mono- to disubstituted derivatives formed during the reaction of ε-amino groups of lysine side chains with α,β-unsaturated compounds. During hydrolysis of the proteins secondary reactions take place that alter this ratio.

The hydrolytic technique was used to prepare a mixture of VIII and IX by hydrolyzing VI for a long period of time. This mixture could be separated on a preparative scale on a Dowex 50 column (Figure 1). Although both compounds were chromatographically pure, they turned out to be extremely hygroscopic and could not be crystallized.

To confirm the suggested structures, acid–base titrations were carried out in an effort to obtain the ratio of amino to carboxyl groups in these compounds. Reproducible titration curves could not be obtained due to the fact that the described decarboxyethylation reactions also occur at the alkaline pH values during the titration of the functional groups.

In summary, synthetic, kinetic, and chromatographic studies demonstrate that amino acids derived from the reaction of ε-amino groups in α-*N*-CBZ-L-lysine, polylysine, reduced bovine serum albumin, and reduced whole wheat gluten with either acrylonitrile or methyl acrylate all have identical elution positions.

Chromatograms of hydrolysates of proteins modified with α,β-unsaturated compounds do not give the true ratio of mono- to disubstituted derivatives because decarboxyethylation reactions occur during the hydrolysis which modify this ratio in favor of the mono derivative. Hydrolysis under alkaline conditions partially removes both substituents and yields lysine.

Finally, these secondary reactions, as well as the elution positions of the various lysine derivatives listed in Table II, should be taken into account when acrylonitrile or other α,β-unsaturated compounds serve as

reagents for subtractive *N*-terminal analyses of peptides and proteins (Fletcher, 1966).

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