Selective Chemical Modification of Arginyl Residues*

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ABSTRACT: Arginine can be converted smoothly to δ-*N*-(2-pyrimidinyl)ornithine by condensation with malonaldehyde in 10 N HCl at 25°. This reaction was applied to bovine plasma albumin, ribonuclease, and lysozyme, and the conversion yield ranged between 83 and 100%. The reaction was accompanied by some side reactions of peptide bond cleavage and disulfide bond interchange because of the strong acid required for the modification. However, the observations on the properties of the modified albumin and ribonuclease tend to indicate that the peptide bond cleavages which had occurred were neither random nor extensive. The peptide bond following the modified arginyl residue was resistant toward trypsin digestion; thus, the reaction may be of use in sequence analysis of proteins. Also, it may be useful for gas chromatography and mass spectrometry studies since the ethyl ester of the modified *N*-benzoylarginine is volatile.

The ε-amino groups of lysyl residues in proteins are modified readily by acylations, but the guanido groups of arginyl residues can be acylated only under vigorous reaction conditions (Zervas *et al.*, 1961). This difference is due to the strong basicity of the guanido group. Nevertheless, there are available a limited number of methods for the modification of arginyl residues. A nonselective method involves the use of formaldehyde which is said to react with the guanido groups in proteins, as well as with the amino and the sulfhydryl groups (Fraenkel-Conrat, 1957). Another, a selective one, utilizes the condensation of guanido groups with 1,2-diketones in alkaline solutions (Toi *et al.*, 1965). Concurrent with the present work, a third method was reported by Yankeelov *et al.* (1966) using biacetyl as a reagent.

The method to be described in the present paper employs a known reaction for the synthesis of 2-aminopyrimidine by the condensation of guanidine with malonaldehyde (Brown, 1962; Kobayashi, 1962). It was found possible to adapt this reaction for the modification of arginyl residues in proteins. Three different proteins were modified by this procedure, and these experimental results are reported below.

Experimental Section

Materials. 1,1,3,3-Tetraethoxypropane which is the ethyl acetal of malonaldehyde was obtained from the Aldrich Chemical Co. Bovine plasma albumin was a product of Armour Co.; bovine pancreatic ribonuclease, trypsin, and egg white lysozyme were products of Worthington Biochemical Corp. Rabbit antiserum for bovine plasma albumin was from Nutritional Biochemicals.

Methods. For amino acid determinations, samples were hydrolyzed in 6 N HCl at 110° for 21 hr. Analyses were made using a Beckman-Spinco amino acid analyzer (Moore and Stein, 1963).

Ribonuclease activity was assayed using the procedure of Kalnitzky *et al.* (1959). Attempts to regenerate the activities of altered ribonuclease samples were carried out using the method of mercaptoethanol-catalyzed disulfide interchange and the method of complete reduction of the disulfide bonds and their subsequent air oxidation (Epstein *et al.*, 1962). The disulfide interchange was carried out in 0.1 M Tris–HCl buffer (pH 8) for 16 hr at about 25°; the protein and mercaptoethanol concentrations were, respectively, 8×10^{-6} and 2×10^{-2} M. The air oxidation of reduced protein (6–20 μg/ml) was carried out in 0.10 M Tris–HCl (pH 8) for 16 hr at about 25°.

Precipitin analyses of bovine plasma albumin were performed using 50 μl each of antigen solution and antiserum. After mixing, the tubes stood at 25° for 15 min, then at 4° for 16 hr. After addition of 0.4-ml aliquots of 0.15 N NaCl, the tubes were centrifuged. The precipitates were washed with a second 0.4-ml aliquot of 0.15 N NaCl, then they were measured by Folin color in a final volume of 2.2 ml. For inhibition analyses, 50 μl of the inhibitor was mixed with 50 μl of antiserum, then after 15 min, 50 μl of the antigen solution was added. After standing overnight at 4°, the amount of precipitate formed was analyzed as described above.

Digestion of albumin (10 mg) by trypsin (0.2 mg) was carried out at pH 8 and 25° in 4 ml of 4 mM Tris plus 2.4 mM HCl plus 1 mM NaCl plus 1 mM $CaCl_2$. The course of digestion was followed by manual addition of 0.1 N NaOH to maintain constant pH. To calculate the number of peptide bonds hydrolyzed, the amount of alkali uptake was divided by 0.7 on the assumption

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that the liberated α-amino group has an average p*K* value of 7.6. For the correction of moisture contents of samples, the number of moles of albumin used was calculated from the results of amino acid analysis and it was assumed that each mole of albumin contained 16.1 residues of histidine (Spahr and Edsall, 1964).

*δ-N-(2-Pyrimidinyl)-*L*-ornithine.* A solution of L-arginine (174 mg, 1 mmole) and 1,1,3,3-tetraethoxypropane (0.27 ml, 1.1 mmole) in 8 ml of 12 N HCl was allowed to stand at 25°. Preliminary rate studies were made by diluting an aliquot at time intervals for ultraviolet spectral determination and for amino acid analysis on the 5-cm column of Spinco amino acid analyzer with the regular pH 5.28 buffer. The reaction was complete after 3 hr, and the solution was rotatory evaporated to dryness at 40°. The residue was stored in a vacuum desiccator over NaOH pellets overnight. After dissolving in 2 ml of water it was applied to a Dowex 50-X8 column (25 × 1 cm) which was equilibrated with 0.3 M HOAc plus 0.1 M pyridine. The column was eluted with a linear gradient produced by 120 ml each of 0.3 M HOAc plus 0.1 M pyridine and 0.3 M HOAc plus 0.2 M pyridine. The flow rate was 60 ml/hr. The column effluents (4 ml/fraction) were analyzed by absorbance at 315 mμ. The effluent (120–180 ml) was pooled and evaporated to dryness. The residue was dissolved in 1 ml of 1 N HCl and 9 ml of ethanol. On addition of pyridine (0.09 ml) the product crystallized out (85 mg, 43% yield). There was a 52% loss during crystallization. After recrystallization it melted at 258–262° with decomposition, melting point block preheated to 240°. Thin layer chromatography on Eastman Chromatogram sheet (type K 301R, silica gel) revealed only one single spot on staining with ninhydrin. It migrated to approximately the same position as that of phenylalanine in a solvent system of *n*-PrOH–28% NH_4OH (67:33). Its optical rotation, $[\alpha]_D^{25}$ +25° (*c* 1.3, 6 N HCl), was similar to that of L-arginine, $[\alpha]_D^{25}$ +26° (*c* 1.3, 6 N HCl). In 0.01 N HCl, its ultraviolet spectrum showed two absorption bands at 230 mμ (ε 17,700) and at 315 mμ (ε 3490).

Anal. Calcd for $C_9H_{14}N_4O_2$: C, 51.41; H, 6.71; N, 26.65. Found: C, 51.63; H, 6.56; N, 26.29.

*α-N-Benzoyl-δ-N-(2-pyrimidinyl)-*L*-ornithine.* Benzoyl-L-arginine (1.11 g, 4 mmoles) and tetraethoxypropane (1 ml, 4.2 mmoles) were dissolved in 20 ml of 12 N HCl. After 16 hr at 25°, the solution was concentrated to a syrup at 40°. The concentration was repeated after addition of water. The residue was dissolved in 15 ml of H_2O and 5 ml of ethanol. On addition of pyridine (0.64 ml, 8 mmoles), the product crystallized. After recrystallization, 0.70 g (56% yield) of product was obtained. It melted at 196–198° with decomposition. Its optical rotation, $[\alpha]_D^{25}$ −7.4° (*c* 2.9, 6 N HCl), was similar to that of benzoyl-L-arginine, $[\alpha]_D^{25}$ −7.5°.

Anal. Calcd for $C_{16}H_{18}N_4O_3$: C, 61.15; H, 5.77; N, 17.82. Found: C, 61.36; H, 5.70; N, 18.03.

*α-N-Benzoyl-δ-N-(2-pyrimidinyl)-*L*-ornithine Ethyl Ester.* A mixture of benzoyl-L-arginine (1.11 g, 4 mmoles) and tetraethoxypropane (1 ml, 4.2 mmoles) in 20 ml of 9.6 N ethanolic HCl was stirred at 25° for 16 hr. The solution was concentrated to a syrup at 40°. After removal of excess HCl by storage in a desiccator over NaOH, 15 ml of concentrated ammonium hydroxide was added. On stirring, a solid precipitate was formed. After filtering and air drying, the solid weighed 1.3 g. It was recrystallized from 140 ml of 14% aqueous ethanol. The yield was 0.74 g (55%). The product melted at 117–119° and its $[\alpha]_D^{25}$ was −14° (*c* 1.3, 6 N HCl). Its infrared spectrum (KBr pellet) showed a sharp ester peak at 1750 cm^{-1} which was absent in that of the benzoylpyrimidinylornithine.

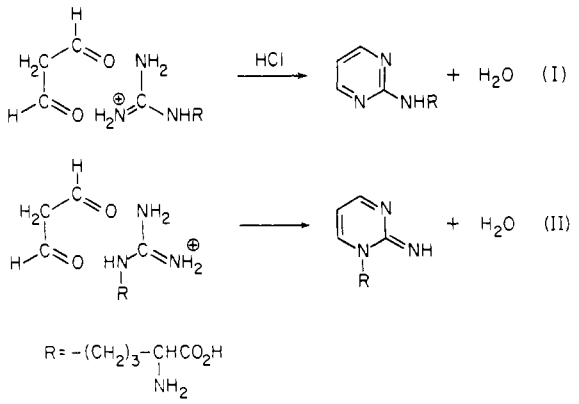
Anal. Calcd for $C_{18}H_{22}N_4O_3$: C, 63.14; H, 6.48; N, 16.36. Found: C, 63.53; H, 6.38; N, 16.47.

Modification of Proteins. The protein (approximately 1.5 μmoles) was dissolved in 1.5 ml of water and then it was added to 8.5 ml of 12 N HCl containing 0.125 ml of tetraethoxypropane (520 μmoles). The course of the reaction could be followed by the absorbancy increase at 315 mμ after making a 1:10 or 1:40 dilution. Appropriate blank corrections were made using a control mixture without protein. After 2 hr, the reaction was terminated by passage through a Sephadex G-25 column (63 × 2.5 cm) at a flow rate of 70 ml/hr. The eluent was 0.2 M HOAc and 8-ml fractions were collected. The first peak containing the protein was pooled and lyophilized.

To investigate the homogeneity of the modified proteins, a sample of about 15 mg was chromatographed on a Sephadex G-100 column (200 × 1 cm). The eluent was 0.4 M $(NH_4)_2SO_4$ plus 0.05 M Tris plus 0.03 M HCl. The flow rate was 10 ml/hr and 2-ml fractions were collected.

Results and Discussion

The reaction of L-arginine with malonaldehyde yields *δ-N*-(2-pyrimidinyl)-L-ornithine with a loss of water as shown in reaction I of Scheme I. The formation of the isomeric product shown in reaction II was not observed. The reaction can be followed conveniently by amino acid analysis or ultraviolet spectrophotometry as the pyrimidinyl product has an ab-



SCHEME I

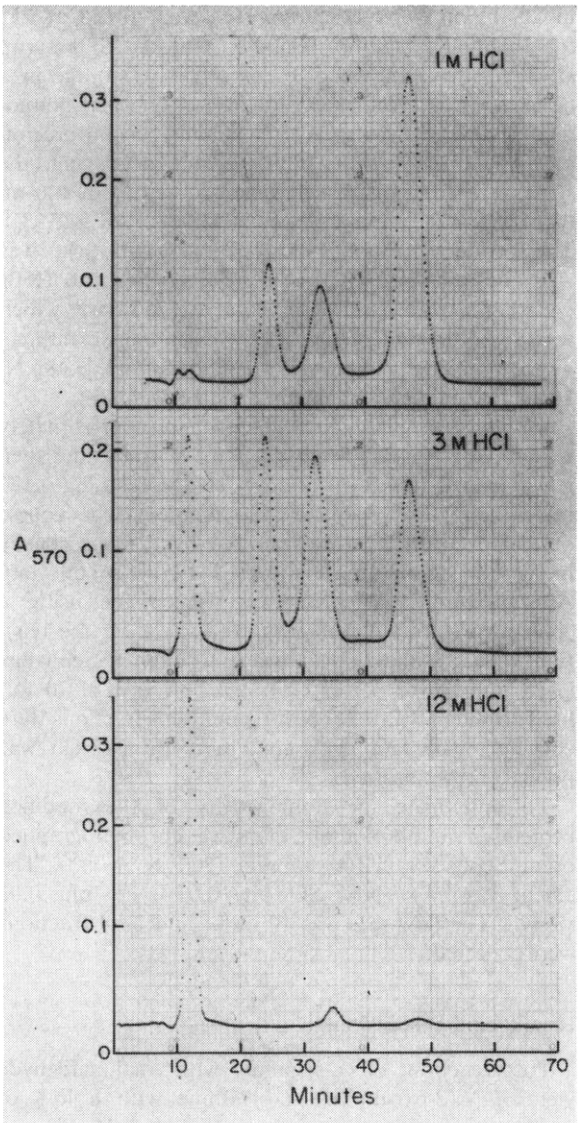


FIGURE 1: Chromatographic analyses of the condensation of arginine (0.10 M) and malonaldehyde (0.105 M) in different concentrations of aqueous HCl after 16 hr at 25°. For 1 and 3 M HCl reaction mixtures, 10-μl aliquots were analyzed. For a 12 M HCl mixture, a 2.5-μl aliquot was used. See Experimental Section for details.

sorption band at 315 mμ in 0.01 N HCl. Figure 1 illustrates the chromatograms obtained with reaction mixtures of L-arginine (0.1 M) and 1,1,3,3-tetraethoxypropane (0.105 M) which is the ethyl acetal of malonaldehyde. The reactions were carried out in 1, 3, and 12 M HCl at 25° for 16 hr. The 12 M HCl mixture gave one large peak of the product eluted at the column front (12 min) and a small peak of arginine eluted at 48 min. The 3 M HCl mixture contained two additional peaks eluted in the middle region. These two peaks are intermediates of the reaction, as analysis after a longer reaction time revealed that their peak heights decreased and that of the product increased.

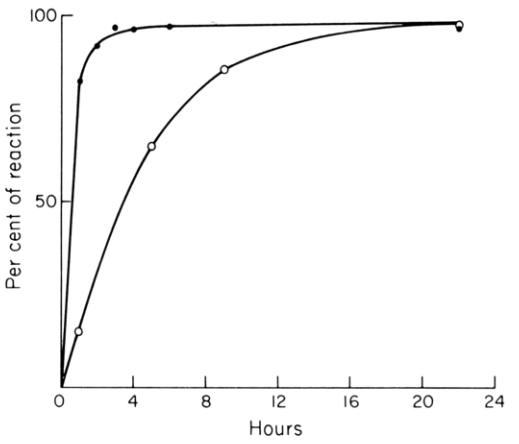


FIGURE 2: The rate of condensation of arginine (0.10 M) and malonaldehyde (0.105 M) in 6 N HCl (●———●) and 12 N HCl (○———○) at 25°. The reaction was followed by absorbancy increase at 315 mμ.

The 1 M HCl mixture contained the intermediates and only a trace of the product. The nature of these intermediates is not known and they do not have absorption bands in the 300-mμ region.

The rates of this reaction in 6 and 12 M HCl as followed by the increase in absorbancy at 315 mμ are shown in Figure 2. The results showed that the rate in 12 M HCl was at least six times faster than that in 6 M HCl and that the reaction in 12 M HCl was complete after 3 hr. This reaction did not take place in neutral solutions and only slowly in alkaline solutions; for these studies the acetal was hydrolyzed first in dilute hydrochloric acid to yield the free malonaldehyde.

The yield of the product as measured by absorbancy was found to be usually several per cent higher than that obtained by amino acid analysis. This was found to be due to esterification of the product by ethanol released from the hydrolysis of the acetal, as short treatment of the reaction mixture with dilute sodium hydroxide at 25° prior to analysis gave identical yields by both methods.

The product was isolated in a crystalline form after chromatography of the reaction mixture on a Dowex 50 column. Its ultraviolet absorption maximum, 315 mμ (ϵ 3490) at pH 2 and 308 mμ (ϵ 2580) at pH 6 or 12, followed closely those reported for the model compound 2-methylaminopyrimidine, 315 mμ (ϵ 3390) at pH 1 and 307 mμ (ϵ 2700) at pH 7. If the product had the structure shown in reaction II of Scheme I, its spectral data would be expected to follow those of 1,2-dihydro-1-methyl-2-iminopyrimidine which has absorption maxima at 301 mμ (ϵ 4260) at pH 7 and at 341 mμ (ϵ 2880) at pH 13 (Brown *et al.*, 1955). From the pH-dependent spectral shift, the pK_a of the substituted δ-amino groups of ornithine was determined to be 3.6, which agrees favorably with that reported for 2-methylaminopyrimidine whose pK_a was reported

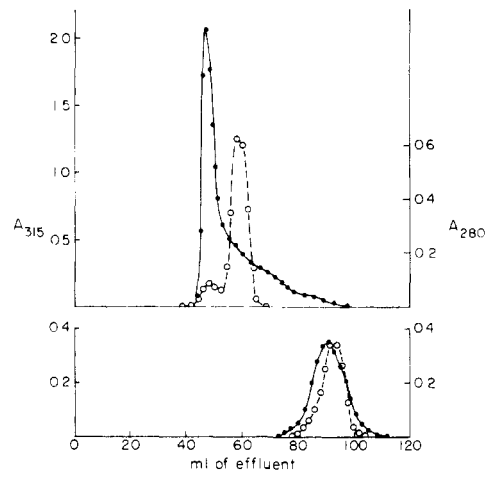


FIGURE 3: Chromatography of ribonuclease and bovine plasma albumin on a column of Sephadex G-100 (200 × 1 cm). The eluent was 0.4 M $(NH_4)_2SO_4$ plus 0.05 M Tris plus 0.03 M HCl. A_{280} of native samples, O----O; A_{315} of modified samples, ●——●. The upper and the lower patterns are for albumin and for ribonuclease, respectively.

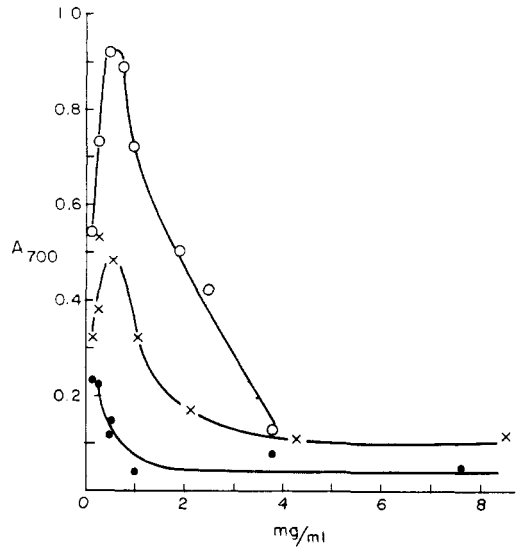


FIGURE 4: Precipitin analyses of bovine plasma albumin with rabbit antiserum. Native albumin, O——O; acid-treated albumin, ×——×; modified albumin, ●—●.

to be 3.8. These observations, as well as that on the hydrolytic behavior of the product to be described below, established its structure as that shown in reaction I of Scheme I.

The product was not stable under the conditions commonly used for the hydrolysis of proteins. Reversal of the condensation and hydrolytic cleavage at the 2 position of the pyrimidine nucleus were found to occur. Amino acid analysis of the product (5 mM) which had been heated in 6 M HCl at 110° for 21 hr gave a mixture of starting material (12%), arginine (22%), and ornithine (66%). The presence of 2-hydroxypyrimidine in the hydrolysate was indicated by its ultraviolet absorption maximum at 308 mμ (ε 4600) in 0.01 N HCl which is in fair agreement with the reported value of 309 mμ (ε 5700) at pH 0 (Brown *et al.*, 1955).

α-*N*-Benzoyl-L-arginine could also be converted into the pyrimidinyl compound. By carrying out the condensation in ethanol, its ethyl ester was isolated and there was no detectable racemization accompanying the reaction. The hydrolysis of this ester (2 mM) at pH 8 was not catalyzed by trypsin (5 μg/ml), in contrast to that of the ethyl ester of benzoylarginine.

The modification reaction was next applied to three proteins: bovine plasma albumin, ribonuclease, and lysozyme. The reaction was carried out in 10 M HCl containing about 0.1 mM protein and 52 mM tetraethoxypropane. After 2 hr at 25°, the excess reagents were removed and the product was lyophilized. Figure 3 illustrates the Sephadex G-100 chromatograms of the modified albumin and ribonuclease as compared with those of native proteins.

Within experimental error, the amino acid composition of each modified protein was the same as that of the native sample, with the exception that there was a decrease in arginine content and the additional presence of ornithine which is a hydrolysis product of the modified arginine derivative. From the decrease in arginine content the conversion yield was estimated to range between 83 and 100%. These results are tabulated in Table I, in which the lysine and the ornithine contents are given together since they were not separated under the conditions used for analysis. Possible loss of tryptophan residues during the reaction was not established.

TABLE 1: Amino Acid Analyses of Modified Proteins.

	Lysine + Ornithine		Arginine		Estd %
	Native	Modified	Native	Modified	Conversion
Bovine plasma albumin[a]	54	65	23	6.4	94
Ribonuclease[b]	10	12	4	0.8	100
Lysozyme[c]	5.7	9.6	10	3.9	83

[a] Calculated on the basis that there are 3.8 residues of methionine/mole of protein (Spahr and Edsall, 1964). [b] Calculated on the basis that there are 12 residues of alanine/mole of protein (Hirs *et al.*, 1956). [c] Calculated on the basis that there are 1.9 residues of methionine/mole of protein (Canfield, 1963).

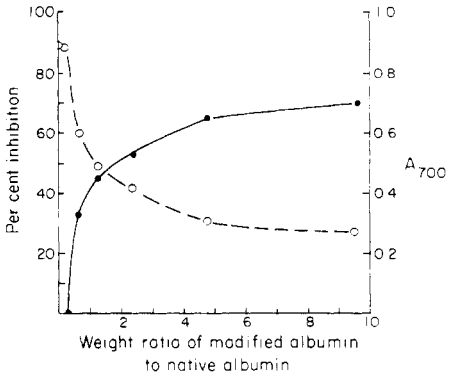


FIGURE 5: Inhibition analyses of modified bovine plasma albumin. The system contained 50 μl of native albumin (0.75 mg/ml), 50 μl of modified albumin of increasing concentrations and 50 μl of antiserum. Per cent inhibition, ●——●, and A_{700} of dissolved precipitate, O---O.

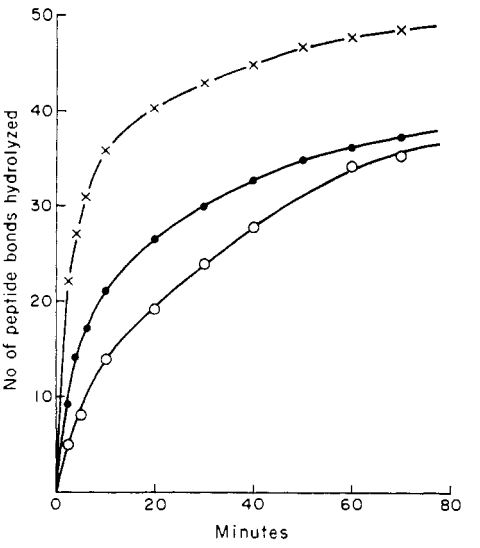


FIGURE 6: Trypsin digestion of bovine plasma albumin. Native albumin, O——O; acid-treated albumin, ×——×; modified albumin, ●---●. The averages of duplicate experiments (±10%) are given.

The modified albumin had lost 80% of its ability to precipitate with a specific antiserum as compared to the native albumin and also its zone of maximum precipitation had changed (Figure 4). However, it had become a good inhibitor of the antialbumin system. With a weight ratio of the inhibitor to albumin of about 5, maximal inhibition of about 70% was achieved (Figure 5). After treatment of bovine plasma albumin under the reaction conditions in the absence of malonaldehyde, it still formed precipitates with its antiserum But the amount of precipitate formed was about one-half of that of native albumin, though its zone of maximum precipitation was not changed (Figure 4). The greater decrease in the ability of the modified albumin to form precipitate as contrasted to the 50% retention of this property by the acid-treated albumin suggested that the replacement of the guanido group side chains with the weakly basic 2-pyrimidinyl groups has caused conformational changes of the protein.

The modified ribonuclease had less than 2% of the original activity. After a sample of ribonuclease had been subjected to the reaction conditions in the absence of malonaldehyde, it had only 5% of the activity. Its activity could be increased to 25% by a thiol-catalyzed disulfide interchange. When the acid-inactivated sample was first reduced with mercaptoethanol, then air oxidized, it had only 2% of the activity. The activity of the modified ribonuclease was not increased by disulfide interchange, or reduction and air oxidation; in fact, it decreased to less than 0.5% following either treatment. A control experiment on the oxidation of reduced native ribonuclease regenerated 80% of its activity.

These control experiments therefore indicated that the side reactions of peptide cleavage and disulfide bond interchange were occurring under the influence of strong HCl required for the modification. However, the observations on the properties of acid-treated albumin and ribonuclease tend to indicate that the peptide bond cleavages which had occurred were neither random nor extensive. A general discussion on the types of peptide bonds which can be cleaved preferentially in the presence of strong acids may be found in a recent review by Hill (1965).

The susceptibilities of the modified, the acid-treated, and the native albumins toward tryptic digestion are illustrated in Figure 6. The modified and the acid-treated albumins were hydrolyzed more readily and to a greater extent than the native albumin. After a digestion time of 70 min, 49 and 37 peptide bonds of the acid-treated and the modified albumins, respectively, were hydrolyzed. Because of the experimental errors involved, this difference of 12 susceptible bonds can be considered in fair agreement with the expected decrease of 21 susceptible bonds as estimated from the amino acid analysis data. Also, these observations showed that the ϵ-amino groups of the lysyl residues did not react with malonaldehyde, or that if reacted, the reaction was readily reversed under the condition of tryptic digestion.

The experiments described above have demonstrated that the arginine residues in proteins can be converted selectively and easily to the pyrimidinylornithine residues, and that these residues are not hydrolyzed by trypsin. Thus, the method may find possible use in sequence studies of proteins for establishing overlapping sequences of tryptic peptides, though there are side reactions of peptide bond cleavage and disulfide bond interchange. Judging from the results obtained with albumin and ribonuclease, the extent of peptide bond cleavage during the reaction is probably not extensive. The disulfide bond interchange reaction does not interfere with sequence analysis studies, since the usual

procedure involves the use of either reduced or oxidized derivatives of proteins.

The present method may also find uses in gas chromatography and mass spectrometry studies of peptides containing arginine residues, as the modified arginine derivative is only weakly acidic and is volatile. For example, the ethyl ester of benzoylpyrimidinylornithine was sublimable at 135° and 0.1 mm. For gas chromatographic analysis of amino acids, arginine has to be converted to its volatile *n*-butyl ester of *N*-tritrifluoroacetyl derivative, but this derivative has a disadvantage that anhydrous conditions are required for its stability (Stalling and Gehrke, 1966). The present modification may be useful for this purpose, as arginine can be converted smoothly through this process to a volatile derivative of ornithine.

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