Reversible Modification of Arginine Residues

APPLICATION TO SEQUENCE STUDIES BY RESTRICTION OF TRYPTIC HYDROLYSIS TO LYSINE RESIDUES*

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SUMMARY

1,2-Cyclohexanedione reacts specifically with the guanidino group of arginine or arginine residues at pH 8 to 9 in sodium borate buffer in the temperature range of 25-40°. The single product, N°,N°-(1,2-dihydroxycyclohex-1,2-ylene)-L-arginine (DHCH-arginine) is stable in acidic solutions and in borate buffers (pH 8 to 9). DHCH-Arginine is converted to N°-adipyl-L-arginine by periodate oxidation. The structures of the two compounds were elucidated by chemical and physicochemical means. Arginine or arginyl residues can be regenerated quantitatively from DHCH-arginine by incubation at 37° in hydroxylamine buffer at pH 7.0 for 7 to 8 hours.

Analysis of native egg white lysozyme and native as well as oxidized bovine pancreatic RNase, which were treated with cyclohexanedione, showed that only arginine residues were modified.

The utility of the method in sequence studies was shown on oxidized bovine pancreatic ribonuclease A. Arginine modification was complete in 2 hours at 35° in borate buffer at pH 9.0 with a 15-fold molar excess of the reagent. The derived peptides showed that tryptic hydrolysis was entirely limited to peptide bonds involving lysine residues, as shown both by two-dimensional peptide patterns and by isolation of the resulting peptides. The stability of DHCH-arginyl residues permits isolation of labeled peptides.

In the last decade a number of bifunctional aldehydes and ketones have been tested for their ability to modify selectively arginine residues in proteins either for the purpose of blocking the hydrolytic action of trypsin at arginyl residues or for modification of arginyl residues in native proteins. Treatment of proteins with benzil (1), cyclohexanedione (2), nitromalondialdehyde (3) in strong alkali, or malonaldehyde in 10 m HCl (4) resulted in quantitative modification of arginine residues. These harsh conditions can lead, however, to a number of side reactions and to irreversible changes (4-7).

Modification with 2,3-butanedione, glyoxal, phenylglyoxal, and cyclohexanedione can be performed under milder conditions (5, 7 11). Unfortunately, all of these procedures may result in significant side reactions with α- or ε-amino groups. To prevent these side reactions the amino groups have to be blocked prior to arginine modification (5, 9, 12-14). An additional problem is caused by the multiple products arising when butanedione (14, 15) or cyclohexanedione (5) is used for arginine modification.

In most cases arginine modification is irreversible, a major disadvantage in protein-sequencing studies. In other instances, such as the reaction of phenylglyoxal with arginine (11), regeneration of arginine occurs spontaneously in neutral and alkaline solutions. This instability of the product places limitations on its use in studies of sequences and for specific labeling. 2,3-Butanedione may also form a product with arginine which regenerates spontaneously to free arginine in the absence of excess reagent (15). The various problems that have been encountered with the above methods impose limitations on their use in protein structural studies.

In this paper we describe the reaction of 1,2-cyclohexanedione with arginine and arginyl residues of proteins at pH 8 to 9 in borate. Apart from the mild conditions used, the method has several additional advantages. Under the conditions specified, cyclohexanedione reacts only with arginine residues. Arginine is converted quantitatively to a single product, DHCH-arginine,1 which is stable in acidic solutions and in borate buffers, allowing isolation and sequence determination of peptides by conventional methods. Under controlled conditions (neutral hydroxylamine or hydrazine solutions), arginine or arginyl residues can be regenerated quantitatively. The experiments described herein were designed to elucidate the chemical nature of the modified arginine and to establish the optimal conditions for its formation and regeneration. The method has been tested in protein sequence work and in enzymological studies, as described herein and in the following paper (16).

1 The abbreviations used are: TPCK, L-1-tosylamido-2-phenyl-ethyl chloromethyl ketone; CHD-arginine, N°-(1,2-dihydroxycyclohex-1,2-ylene)-L-arginine; N°-adipyl-L-arginine; CHD, 1,2-cyclohexanedione.
EXPERIMENTAL PROCEDURES

Materials—1,2-Cyclohexanedione, 1,2-cyclohexanedione dioxime, and 1-amino-1-cyclopentanecarboxylic acid (Aldrich), adipic acid (Sigma), hydroxylamine hydrochloride (Eastman), anhydrolysin (Matheson, Coleman, and Bell), phenanthrenequinone, sodium metaperiodate (Baker), and L-arginine hydrochloride (Merek) were analytical reagent grade. Crystaline egg white lysozyme was obtained from Armour Laboratories and bovine pancreatic ribonuclease A from Sigma. Carboxypeptidase B and TPCK-treated trypsin were purchased from Worthington Laboratories.

Paper Electrophoresis and Paper Chromatography—High-voltage electrophoresis was performed at pH 1.9, 3.0, 4.7, 5.0, and 8.0. Paper chromatography was carried out in 1-butanol-pyridine-acetic acid-water (15:10:3:12) or 1-butanol-acetic acid-water (40:6:15). Both methods employed Whatman No. 3MM papers. Two-dimensional peptide patterns were obtained by 1-butanol-pyridine-acetic acid-water chromatography in one dimension and electrophoresis at pH 1.9 in the other.

Amino Acid Analyses—Analysis of the products of reaction between arginine and 1,2-cyclohexanedione in different media was achieved by paper electrophoresis and paper chromatography. The relative amounts of the different products that were present were determined by analysis on the short column of a Beckman model 121 Automatic Amino Acid Analyzer. Protein samples were estimated by cutting out the ninhydrin-stained spots and eluting with 50% ethanol. Concentration was determined by measurement of the absorption at 500 nm.

Experiments with L-arginine (0.05 m) and 1,2-cyclohexanedione (0.06 m) were studied in the following buffer solutions (0.2 m): A-ethylmorpholine at pH 7.5, sodium borate at pH 10.0 and 11.0. Solutions of NaOH (0.2 and 0.5 m) and 30%/acetic acid, and 0.01 M HCl were used to adjust the pH. The rate of decomposition of modified arginine (0.1 m) in 0.5 m hydroxylamine at pH 7.0 was followed by the quantitative determination of arginine and DHCH-arginine on the short column of the amino acid analyzer.

Oxidation of DHCH-arginine was performed with 0.02 M sodium metaperiodate in 0.2 M sodium acetate at pH 4.0. Reaction was allowed to proceed for 16 hours at 0°C in the dark. Periodate consumption was determined at 1:2, 1:1, 2:1, and 4:1 periodate to DHCH-arginine ratios by titration with sodium thiosulfate in 0.1 M NaOH solution. The dioxime formed a red complex with sodium thiosulfate in acidified solution.

Infrared Spectra—Infrared spectra were obtained on potassium bromide wafers with a Perkin-Elmer 237B Grating Infra-Red spectrophotometer. NMR spectra of DHCH-arginine and adipylarginine were determined in 0.2 M solutions (D2O) by use of a Varian HA 100 NMR spectrometer.

Electrophoresis—The peptides were separated on 7-cm polyacrylamide gels, using a Beckman model 121 Automatic Amino Acid Analyzer.

RESULTS

Reaction of L-arginine with 1,2-Cyclohexanedione—In preliminary experiments the reaction of L-arginine (0.05 m) and 1,2-cyclohexanedione (0.06 m) was studied in the following buffer solutions (0.2 m): A-ethylmorpholine at pH 7.5, sodium borate at pH 10.0 and 11.0. Solutions of NaOH (0.2 and 0.5 m) and 30%/acetic acid, and 0.01 M HCl were used to adjust the pH.

The rates of reaction in 0.2 M sodium borate buffer at pH 9.0 were determined at 35°C, 30°C, 25°C, and 0°C with 0.15 m 1,2-cyclohexanedione and 0.025 m L-arginine. Aliquots were removed into cold 30% acetic acid for analysis by paper chromatography and paper electrophoresis.

Preparation of DHCH-arginine—L-Arginine-HCl (1.05 g, 5 mmol) was dissolved in 100 ml of freshly prepared 0.2 M sodium borate buffer at pH 9.0 and 0.07 g (6 mmol) of 1,2-cyclohexanedione were added; the reaction mixture was kept under N2 at room temperature. Aliquots were removed at intervals and diluted into cold 30% acetic acid for analysis by paper chromatography and paper electrophoresis.

Preparation of N2,N2'-dihydrazide-1,2-glyl-L-arginine Hydrochloride (DHCH-arginine)—L-Arginine-HCl (1.05 g, 5 mmol) was dissolved in 100 ml of 0.01 M HCl. The preparation was desalted and purified by gel filtration on Sephadex G-10 columns (2 × 150 cm) in 0.01 M HCl at a flow rate of 40 ml per hour. Five-milliliter fractions were collected and 5-ml aliquots were removed for analysis by high voltage electrophoresis at pH 4.7 and paper chromatography. Fractions containing the pure DHCH-arginine were pooled and lyophilized. The hygroscopic material was stored at -20°C under vacuum or in 0.01 M HCl.

The pH of the reaction mixture of 1,2-cyclohexanedione and arginine has a dramatic effect on the nature and number of products formed, as shown in Fig. 1. As described previously (2), in strong alkali a single major product, CHD-arginine, is formed, whereas at slightly less alkaline pH additional products appear, e.g. at pH 10 to 11 (trimethylamine buffers) as many as five different products are present in substantial amounts. At pH 7.5 to 9.0, however, only a single product is formed. Since homogeneity of product is a desirable attribute of modification reactions, we investigated in considerable detail the conditions required for the quantitative formation of this product.

Tris buffers cannot be used inasmuch as Tris reagents with 1,2-
cyclohexanedione, thereby preventing arginine modification. Initial experiments showed that the rate of reaction was highest at pH 9.0 in sodium borate buffer. The rate of reaction in this buffer was studied at different temperatures. As shown in Fig. 2, the reaction is first order with respect to disappearance of arginine. Furthermore, there is almost a 10-fold decrease in the half-time of the reaction on going from 25-40° (Fig. 2, inset) with no change in the nature of the reaction product, as demonstrated by various chromatographic studies.

Fig. 1. Paper chromatogram (1-butanol-pyridine-acetic acid-water (15:10:3:12)) of the products of reaction between 1,2-cyclohexanediene and arginine in: N-ethylmorpholine, pH 7.5; sodium borate, pH 9.0; trimethylamine, pH 11.0; 0.2 N NaOH and 0.5 N NaOH. Reaction was performed as described under "Experimental Procedures." The samples were run after 7 hours of reaction. Std., standards.

Fig. 2. First order plot of the rate of reaction of cyclohexanediene with arginine at different temperatures. The inset shows the change of half-time of reaction with temperature.

Fig. 3. Electrophoretic mobility of adipylarginine, DHCH-arginine in the presence and absence of borate at different pH values. Mobilities are expressed in per cent of mobility of arginine.

Description of DHCH-Arginine

Unlike CHD-arginine, the product is a strong base; its relative electrophoretic mobility (76% of that of arginine) is the same from pH 1.9 to pH 8.0 as shown in Fig. 3. Titration of the modified group was not feasible, since at higher pH values the product undergoes rapid decomposition and secondary reactions (see below).

DHCH-Arginine is eluted from the short column of the amino acid analyzer between ammonia and arginine, further indicating its basicity. Unlike CHD-arginine (2), the determination of DHCH-arginine does not require special conditions for separation from other amino acids. Based on modification and regeneration experiments (hydroxylamine), the ninhydrin color value of DHCH-arginine is identical with that of arginine. In paper chromatography (1-butanol-pyridine-acetic acid-water) the RF value of DHCH-arginine is 0.34 as compared to 0.23 for arginine and 0.46 for CHD-arginine (Fig. 1).

Upon hydrolysis in 6 N HCl at 110° for 24 hours the product is destroyed. There is an 18 to 20% regeneration of arginine; the remainder is converted to unknown basic products that are not eluted from the amino acid analyzer under normal conditions. If acid hydrolysis is carried out in the presence of excess mercaptoacetic acid (20 μl), DHCH-arginine is converted to a neutral product, which is eluted after the aromatic amino acids on the short column. Since under these conditions no arginine regeneration occurs, the amount of arginine in modified peptides and proteins was always determined after hydrolysis in the presence of mercaptoacetic acid.

Alkaline hydrolysis in 2.5 M NaOH at 110° for 24 hours results in the formation of ornithine, citrulline, and 1-amino-1-cyclopentane-carboxylic acid as identified by paper chromatography, paper electrophoresis, or amino acid analysis. Formation of 1-amino-1-cyclopentane-carboxylic acid is due to the fact that in strong alkali, DHCH-arginine forms CHD-arginine (see below), which has been shown to yield 1-amino-1-cyclopentane-carboxylic acid (2).

Studies on the stability of DHCH-arginine in neutral and weakly alkaline solutions indicate that it decomposes to regenerate cyclohexanedione and arginine (Scheme I). The secondary reactions yield the same product (or products) as those observed when the arginine modification is carried out under the same conditions. Thus in triethylamine buffer at pH 11.0 the five characteristic products appear (see Fig. 1); in 0.5 M NaOH CHD-arginine...
arginine is formed. In the latter case the progress curve clearly shows the secondary reaction (Fig. 4).

As expected, at pH 8.0 to 9.0, the liberated arginine does not yield new products. The rate of decomposition to free arginine increases with pH and temperature and is also strongly dependent on the chemical nature of the buffer. The decomposition is more rapid in buffers that react with 1,2-cyclohexanedione (Tris, hydrazine, hydroxylamine) and buffers that are strong nucleophiles (hydroxylamine, hydrazine, Tris, ammonium). This is probably due to nucleophilic catalysis of decomposition and trapping of liberated cyclohexanedione. Trapping of cyclohexanedione explains why no secondary products appear at pH 10 to 11, when hydroxylamine or hydrazine solutions are used. Since the pH of these solutions does not seem to affect greatly the rate of decomposition, we used hydroxylamine buffer at pH 7.0 for regeneration of arginine. On customary semilogarithmic plots, the decomposition of DHCH-arginine is first order. The half-life of DHCH-arginine in 0.5 M hydroxylamine at pH 7.0 and 37° is 100 min (Fig. 5).

DHCH-Arginine is remarkably stable in sodium borate buffers at pH 8.0 to 9.0, since no arginine is liberated over a 24-hour period at room temperature or 37°, whereas in sodium phosphate buffer at pH 8.0 there is 10% (room temperature) and 40% (37°) regeneration in 24 hours. The reason for this unusual stability is that borate forms a complex with the product, thereby stabilizing it (see below). The exceptional stability of DHCH-arginine in borate buffer also explains the high rate of formation of this product in this buffer. DHCH-Arginine is also stable in acidic solution, e.g. no detectable regeneration occurs in 30% acetic acid at 25–37° over a 24-hour period.

Characterization of DHCH-Arginine

Complex formation of DHCH-arginine with borate was shown by a change in its RF value (0.27 instead of 0.35) when boric acid-impregnated papers (21) were used for paper chromatography in 1-butanol-pyridine-acetic acid-water; the RF values of other amino acids were unaffected. In buffers containing 0.1 M boric acid the relative electrophoretic mobility of DHCH-arginine, in agreement with the introduction of a negative charge, decreases significantly (Fig. 3, and Scheme I). The effect of complex formation on the stability and rate of formation of DHCH-arginine has been discussed above.

DHCH-Arginine does not react with 2,4-dinitrophenylhydrazine (22), and is not stabilized by treatment with sodium borohydride.

DHCH-Arginine reacts with 1 molecule of periodate, as determined by sodium thiosulfate titration of unreacted periodate, to yield N7-adipylarginine (Scheme II). The latter is eluted from the short column of the analyzer at the position of lysine; thus special conditions are required for its resolution. The ninhydrin color value of adipylarginine is 115% of that of arginine. Its RF value is 0.39 on paper chromatography with 1-butanol-pyridine-acetic acid-water.

In accord with the presence of an amide bond between adipic acid and one of the guanidino nitrogen atoms of arginine, on hydrolysis in 6 N HCl at 110° for 24 hours, adipylarginine is hydrolyzed quantitatively to yield arginine and adipic acid. Adipic acid was identified by paper chromatography in 1-butanol-acetic acid-water and 1-butanol-pyridine-acetic acid-water, as well as by electrophoresis at pH 3.6, 4.7, and 6.5. The mobilities were in all instances identical with those of an authentic sample of adipic acid. Bromphenol blue was used to detect the acid.

Adipylarginine is stable in acidic solutions (pH lower than 5) at room temperature, but is hydrolyzed to arginine and adipic acid in alkaline solutions (pH above 7).

Structure of DHCH-Arginine—DHCH-Arginine is the addition product of arginine and 1,2-cyclohexanedione, as shown in Scheme
I. Formation of a borate complex and sensitivity to periodate oxidation indicate the presence of vicinal cis-hydroxyl groups in the molecule. Lack of reaction with phenylhydrazine and borohydride are also in agreement with this structure. Dissociation of DHCH-arginine to arginine and cyclohexanedione on removal of borate also shows that the cyclohexane ring is intact in the molecule. Dihydroxyl addition products analogous to DHCH-arginine have been described for the reaction of butanedione with benzamidine (23, 24) and glyoxal or benzil with urea (25). While our work was in progress a similar structure was suggested for the reaction of monomeric 2,3-butanedione with arginine (26), but proof of the structure was not presented.

As predicted from the structure of DHCH-arginine shown in Scheme I, periodate oxidation yields adipylarginine (Scheme II). NMR and infrared spectra (see below) indicate that the product formed on periodate oxidation is $N^7$-adipyl-$L$-arginine (Structure II) rather than $N^7$, $N^8$-adipyl-$L$-arginine (Structure I). The cyclic intermediate (I) is presumably rapidly hydrolyzed because of the strain inherent in this structure, to yield Structure II. The change in electrophoretic mobility of adipylarginine at acidic pH values is in good agreement with the pK$_1$ = 4.43 of adipic acid (Fig. 3). Furthermore, on standing in alkaline solutions, arginine was released from adipylarginine without any evidence for the formation of an intermediate, as expected from the postulated structure with one amide bond.

Comparison of NMR spectra of DHCH-arginine and adipylarginine shows that on periodate oxidation an asymmetrical structure is formed (Fig. 6). The chemical shifts for the peaks arising after periodate oxidation are in agreement with published values for related structures: Sadtler Standard Spectra, No. 4406 (adipic acid); Nos. 5126, 5127, and 5184 (N-acylguanidino compounds (27)).

The infrared spectrum of DHCH-arginine shows a characteristic peak at 1055 cm$^{-1}$, probably corresponding to vicinal hydroxyl groups. This peak is absent from the spectrum of adipylarginine which also possesses peaks at 1680 and 1720 cm$^{-1}$; these are compatible with reported values for adipic acid (SSS No. 281) and N-acylguanidino compounds (SSS Nos. 23182, 23185).

![Fig. 6. NMR spectra of DHCH-arginine and adipylarginine. The arrows indicate the assignment of the various peaks to protons in the two compounds. Values in parentheses represent the number of protons.](http://www.jbc.org/)

![Scheme II](http://www.jbc.org/)

![Scheme II](http://www.jbc.org/)
Modification of Arginine Residues in Proteins

Amino acid analysis of proteins treated with 1,2-cyclohexanedione and peptides obtained from modified RNase indicate that only arginine residues are modified under the conditions employed (Tables I and II) (16). As described previously (5), lysine residues may react with 1,2-cyclohexanedione to form a yellow product (440 nm) when longer reaction times are employed. Under the conditions specified in our work, this side reaction was not observed. Treatment of neutral pH or in strong alkali. Under the conditions specified for destruction during acid hydrolysis. The His-Ile-Ile sequence is hydrolyzed very slowly.

Values for threonine and serine are corrected by 5 and 10%, respectively, for destruction during acid hydrolysis.

Values after 96 hours of acid hydrolysis. The His-Ile-Ile sequence is hydrolyzed very slowly.

Arginine was determined first in the modified peptide; the second value given is for a sample of peptide which was hydrolyzed after treatment with hydroxylamine.

Each peptide has been purified by a single step of purification by paper electrophoresis or paper chromatography; yields are uncorrected for pooling of fractions, removal of aliquots, etc.

### Table I

Amino acid composition of 1,2-cyclohexanone-modified proteins

| Amino acid | Modified oxidized RNase | Modified lysozyme |
|------------|-------------------------|--------------------|
| Aspartic acid | 15.4(1) | 21.6(21) |
| Threonine | 6.9(10) | 7.4(7) |
| Serine | 14.2(15) | 8.6(10) |
| Glutamic acid | 12.3(2) | 5.3(6) |
| Proline | 4.2(4) | n.d. |
| Glycine | 3.5(5) | 12.5(15) |
| Alanine | 12.1(3) | 11.9(12) |
| Cysteine | 7.5(8) | n.d. |
| Valine | 8.9(9) | 5.7(6) |
| Methionine | 4.0(4) | 2.0(2) |
| Isoleucine | 6.4(2) | 2.0(2) |
| Leucine | 2.2(2) | 8.2(6) |
| Tyrosine | 5.9(6) | 2.9(2) |
| Phenylalanine | 3.0(3) | 2.9(2) |
| Tryptophan | 5.1(6) | |
| Lysine | 10.2(10) | 5.9(6) |
| Histidine | 7.9(4) | 1.2(1) |
| Arginine | <0.1(4) | 1.6(11) |

All values are uncorrected; n.d., not determined. Values in parenthesis represent the number of residues in the sequence.

### Table II

Amino acid composition of tryptic peptides of CHD-treated RNase

| Amino acid | T(c)1 | T(c)2 | T(c)3 | T(c)4 | T(c)5 | T(c)6 | T(c)7 | T(c)8 | T(c)9 |
|------------|------|------|------|------|------|------|------|------|------|
| Aspartic acid | 5.4(1) | 5.2(1) | 5.1(1) | 5.0(1) | 4.9(1) | 4.8(1) | 4.7(1) | 4.6(1) | 4.5(1) |
| Threonine | 5.6(5) | 5.5(5) | 5.4(5) | 5.3(5) | 5.2(5) | 5.1(5) | 5.0(5) | 4.9(5) | 4.8(5) |
| Serine | 5.8(8) | 5.7(8) | 5.6(8) | 5.5(8) | 5.4(8) | 5.3(8) | 5.2(8) | 5.1(8) | 5.0(8) |
| Glutamic acid | 5.1(5) | 5.0(5) | 4.9(5) | 4.8(5) | 4.7(5) | 4.6(5) | 4.5(5) | 4.4(5) | 4.3(5) |
| Proline | 5.2(2) | 5.1(2) | 5.0(2) | 4.9(2) | 4.8(2) | 4.7(2) | 4.6(2) | 4.5(2) | 4.4(2) |
| Glycine | 5.3(3) | 5.2(3) | 5.1(3) | 5.0(3) | 4.9(3) | 4.8(3) | 4.7(3) | 4.6(3) | 4.5(3) |
| Alanine | 5.4(4) | 5.3(4) | 5.2(4) | 5.1(4) | 5.0(4) | 4.9(4) | 4.8(4) | 4.7(4) | 4.6(4) |
| Cysteine | 5.5(5) | 5.4(5) | 5.3(5) | 5.2(5) | 5.1(5) | 5.0(5) | 4.9(5) | 4.8(5) | 4.7(5) |
| Valine | 5.6(6) | 5.5(6) | 5.4(6) | 5.3(6) | 5.2(6) | 5.1(6) | 5.0(6) | 4.9(6) | 4.8(6) |
| Methionine | 5.7(7) | 5.6(7) | 5.5(7) | 5.4(7) | 5.3(7) | 5.2(7) | 5.1(7) | 5.0(7) | 4.9(7) |
| Isoleucine | 5.8(8) | 5.7(8) | 5.6(8) | 5.5(8) | 5.4(8) | 5.3(8) | 5.2(8) | 5.1(8) | 5.0(8) |
| Leucine | 5.9(9) | 5.8(9) | 5.7(9) | 5.6(9) | 5.5(9) | 5.4(9) | 5.3(9) | 5.2(9) | 5.1(9) |
| Tyrosine | 6.0(10) | 5.9(10) | 5.8(10) | 5.7(10) | 5.6(10) | 5.5(10) | 5.4(10) | 5.3(10) | 5.2(10) |
| Phenylalanine | 6.1(11) | 6.0(11) | 5.9(11) | 5.8(11) | 5.7(11) | 5.6(11) | 5.5(11) | 5.4(11) | 5.3(11) |
| Lysine | 6.2(12) | 6.1(12) | 6.0(12) | 5.9(12) | 5.8(12) | 5.7(12) | 5.6(12) | 5.5(12) | 5.4(12) |
| Histidine | 6.3(13) | 6.2(13) | 6.1(13) | 6.0(13) | 5.9(13) | 5.8(13) | 5.7(13) | 5.6(13) | 5.5(13) |
| Arginine | 6.4(14) | 6.3(14) | 6.2(14) | 6.1(14) | 6.0(14) | 5.9(14) | 5.8(14) | 5.7(14) | 5.6(14) |

Yield (%)

| Yield (%) | 32.2 | 40.5 | 16.7 | 16.1 | 24.1 | 34.0 | 7.6 | 22.9 | 15.2 | 57.4 | 59.3 |

Values for threonine and serine are corrected by 5 and 10%, respectively, for destruction during acid hydrolysis.

Values after 96 hours of acid hydrolysis. The His-Ile-Ile sequence is hydrolyzed very slowly.

Arginine was determined first in the modified peptide; the second value given is for a sample of peptide which was hydrolyzed after treatment with hydroxylamine.

Each peptide has been purified by a single step of purification by paper electrophoresis or paper chromatography; yields are uncorrected for pooling of fractions, removal of aliquots, etc.
vicinal cis-hydroxyl groups in the molecule, it forms a complex reactions with proteins (28), in the absence of Cu²⁺ or O₂ (29).

The blocking group can be removed by hydroxylamine at pH 7.0, is stable in acidic solutions and in borate buffers at pH 8 to 9. Slightly alkaline solutions to form a single new amino acid derivative.

In a preparative experiment the tryptic peptides obtained from modified RNase were fractionated by gel filtration and all the peptides were purified by paper chromatography or paper electrophoresis (Fig. 8). The peptides derived from the cyclohexanediol-treated RNase are designated by the prefix T(c) and numbered consecutively from the NH₂-terminal end. Analysis of the peptides present in the tryptic digest confirms that cleavage had taken place only at lysine residues (Table II). Partial cleavage at lysine residues was observed in both control and cyclohexanediol-treated RNase and in the isolation of T(c)8+4 and T(c)6+7.

In the present study the peptides were purified without prior regeneration of arginine to show that this does not occur during purification procedures at acidic pH values. In subsequent studies of protein sequences, it may be desirable to regenerate arginine residues after the tryptic hydrolysis, depending on the conditions used for regeneration of arginine (pH 7.0, 37° for 6 to 7 hours), hydroxylamine does not produce other reactions with proteins (28), in the absence of Cu²⁺ or O₂ (20).

**DISCUSSION**

1,2-Cyclohexanediol reacts with free arginine in neutral and slightly alkaline solutions to form a single new amino acid derivative, N⁷⁺N⁸⁺(1,2-dihydroxy-cyclohex-1,2-ylene)-L-arginine, which is stable in acidic solutions and in borate buffers at pH 8 to 9. The blocking group can be removed by hydroxyamine at pH 7.0, with quantitative recovery of arginine.

The structure of DHCH-arginine was elucidated by chemical and physiochemical methods. Consistent with the presence of vicinal cis hydroxyl groups in the molecule, it forms a complex with borate, and the carbon-carbon bond between the hydroxyl groups is cleaved by periodate oxidation, yielding N⁷-adipyl-arginine. Adipylarginine yields quantitatively adipic acid and arginine on hydrolysis. NMR and infrared spectra of both DHCH-arginine and adipylarginine are in agreement with the predicted structures. Evidence for an intact cyclohexane ring comes from the fact that DHCH-arginine is slowly hydrolyzed to yield cyclohexanedione and arginine on removal of borate. Analogous reactions have been described between dicarbonyl compounds and benzamide or urea (23–25).

1,2-Cyclohexanediol treatment of proteins results in specific modification of arginyl residues, restricting the action of trypsin to hydrolysis at lysine residues. The homogeneity of the product and its stability in different solutions simplify subsequent analytical procedures. The tryptic peptides thus obtained can be subjected to secondary digestion with trypsin after removal of the blocking groups by hydroxyamine at pH 7.0. In this manner the reversible blocking of arginine residues provides a convenient method for sequence studies, supplementing the various methods for selective, reversible modification of lysine residues.

In the absence of methods for the specific modification of arginine residues under mild conditions, very little has been known of the role of arginine residues in protein structure and function. The mild conditions used in the present procedure, the homogeneity of product, specificity of reaction, reversibility under mild conditions, etc., suggest its possible usefulness as a probe for ascertaining the role of arginine residues in specific proteins. Some first experiments with native lysozyme and RNase are reported in the following paper (16).

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