Alkylation and Identification of the Histidine Residues at the Active Site of Ribonuclease

ARTHUR M. CRESTFIELD, WILLIAM H. STEIN, AND STANFORD MOORE

From The Rockefeller Institute, New York 21, New York

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The reactions of iodoacetate and bromoacetate with ribonuclease have already provided some information on the relationship between the chemical structure and the catalytic activity of the enzyme. It is the purpose of this and the following paper to extend this information. In earlier studies, Gundlach, Stein, and Moore (1) showed that although alkylation at lysine, methionine, or histidine residues could occur, the most specific inactivation by iodoacetate involved predominantly the formation at pH 5.5 to pH 6 of a derivative which contained a single residue of 1 (or 3) carboxymethylhistidine. Independently, Barnard and W. D. Stein (2, 3) studied a similar type of reaction in which C14-labeled bromoacetate was the alkylating agent. Upon oxidation of all of the protein in the reaction mixture, hydrolysis with chymotrypsin, and separation of the products by paper electrophoresis, Barnard and Stein found a single radioactive peptide; from the amino acid composition of the peptide they concluded that the histidine which had been alkylated in ribonuclease was the one at position 119.

Gundlach et al. also observed the formation at pH 5.5 of a small amount of a second inactive derivative which had a slower rate of travel on Amberlite IRC-50. The present communication is concerned with the characterization of the two products of the reaction, the establishment of the position of the amino acid residues altered in each instance, and the determination of which of the two structural isomers (1-carboxymethyl- or 3-carboxymethylhistidine) is formed when a given imidazole ring in ribonuclease was alkylated by iodoacetate.

EXPERIMENTAL PROCEDURE

Ribonuclease A—The enzyme was prepared as previously described (4). For the present experiments, it was important to remove polyvalent anions by the procedure of Dixon ((5); cf. (4)).

Assay of Ribonuclease—The activity of ribonuclease was usually determined with 2′, 3′-cyclic cytidylic acid as substrate, as already described (6); occasionally the cyclic cytidylate was replaced by an equal weight of ribonuclease A dissolved in 0.1 ml of water. The mixture was brought to pH 5.5 by the addition of about 1 ml of 0.2 M phosphate buffer at pH 6.47 used for the chromatography. The phosphate ions in the buffer inhibit further alkylation, and the samples may be stored at 4° until chromatographed.

Preparation of Products of Alkylation at pH 5.5—Since polyvalent ions inhibit the alkylation reaction (cf. the accompanying paper (11)), it is essential that, before use, the ribonuclease be rigorously desalted in the manner already described. It is not necessary, however, to begin with purified ribonuclease A, provided that the sample employed is free from components that emerge from the IRC-50 columns after ribonuclease A and near the positions of the carboxymethylhistidine derivatives. Pure products have been isolated after alkylation of several different commercial samples of ribonuclease that had merely been desalted.

The relative quantities and concentrations of reactants in the alkylation procedure can be varied considerably for preparative purposes. For example, if 500 mg of ribonuclease A dissolved in water are brought to pH 5.5 with NaOH and are diluted to a volume of about 7.5 ml, and if 50 mg of iodoacetate in 7.5 ml of water (adjusted to pH 5.5) are added to the solution, alkylation will be completed in a working day. After the mixture has stood at 25° for about 6 hours, 200 μl are analyzed chromatographically. At this time there is usually about 20% residual ribonuclease A and most of the protein will appear in the more retarded peaks shown in Fig. 1. Between the eighth and ninth hours the residual ribonuclease A will have almost disappeared, at which point the reaction mixture is applied to the column (4 × 30 cm) of IRC-50 of the type used in the preparation of ribonuclease A (4). The chromatographic fractionation is carried out as with ribonuclease A. The pooled fractions containing each of the two main products are best stored frozen. The solutions may be concentrated by ultrafiltration or the ionic environment may be changed by the use of columns of Sephadex...
G-25 as described for ribonuclease A. If necessary, the major product may be desalted by the procedure of Dixon (4, 5), followed by gel filtration on Sephadex G-25 (4). This procedure is not recommended for the second product because of its low concentration. This minor component is desalted either by ultrafiltration or on Sephadex G-25. The yield of the major product from 500 mg of ribonuclease A is of the order of 350 to 400 mg. The yield of the minor product is 25 to 30 mg. Amino acid analyses of the isolated products were carried out after reduction and carboxymethylation of each derivative (cf. Table 1). This procedure converts cystine to S-carboxymethylcysteine, which moves rapidly on the amino acid analyzer, thereby making it possible to detect 3-carboxymethylhistidine, which emerges in the same position as mesocystine (1).

Location of Position of Carboxymethylated Histidine Residue in Each of the Alkylated Products—The derivatives were reduced with mercaptoethanol, the -SH groups were carboxymethylated with iodocetic acid, and the products were hydrolyzed by trypsin according to the procedures described in an earlier paper (10). Samples of the 8-hour hydrolysates were chromatographed on the 15-cm column of the amino acid analyzer (Fig. 2). The buffers normally employed with the instrument (10) were used as eluents. The remainder of the hydrolysate was frozen. A preliminary separation of a given peptide was accomplished by repeating the chromatography with the aid of a column (0.9 X 15 cm) and a fraction collector. A quantity corresponding to the hydrolysate from about 5 mg of protein was chromatographed. Samples of 0.2 ml from the effluent fractions were analyzed after alkaline hydrolysis (4). BRJ 35 and thiodiglycol were omitted from the buffers so that preliminary amino acid analyses could be carried out without desalting the pooled fractions.

The analyses, together with the data of Hirs, Moore, and Stein (13) and of Smyth, Stein, and Moore (15, 16) on the sequence of ribonuclease, indicated that the predominant carboxymethylhistidine-containing peptide in the new position on the sequence of ribonuclease, indicated that the predominant carboxymethylhistidine-containing peptide in the new position on the enzyme was obtained after a 3-hour solution of ribonuclease A had been alkylated at 25° and pH 6.47 as eluent (12).

The results are expressed as the number of amino acid residues per molecule. For calculation of the molar ratios, the average of the micromoles of glutamic acid and alanine found was assumed to represent 12.0 residues, the known number of each of these residues in ribonuclease A (4, 13). Each column represents the results of a single analysis of a 22-hour hydrolysate.

Table I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>RCM-RNase</th>
<th>RCM-1-carboxymethylhistidine-119-RNase</th>
<th>RCM-3-carboxymethylhistidine-12-RNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>14.9</td>
<td>14.8</td>
<td>15.2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>12.2</td>
<td>12.0</td>
<td>12.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.98</td>
<td>3.15</td>
<td>3.09</td>
</tr>
<tr>
<td>Alanine</td>
<td>11.9</td>
<td>12.0</td>
<td>11.9</td>
</tr>
<tr>
<td>Valine</td>
<td>8.73</td>
<td>8.58</td>
<td>8.50</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.07</td>
<td>2.02</td>
<td>1.85</td>
</tr>
<tr>
<td>Isoleucine&lt;</td>
<td>2.31</td>
<td>2.26</td>
<td>2.11</td>
</tr>
<tr>
<td>Serine</td>
<td>13.2</td>
<td>13.0</td>
<td>13.6</td>
</tr>
<tr>
<td>Threonine&lt;</td>
<td>9.42</td>
<td>9.40</td>
<td>9.90</td>
</tr>
<tr>
<td>Methionine&lt;</td>
<td>3.80</td>
<td>3.90</td>
<td>3.71</td>
</tr>
<tr>
<td>S-Carboxymethylcysteine</td>
<td>8.08</td>
<td>7.96</td>
<td>8.00</td>
</tr>
<tr>
<td>Proline</td>
<td>4.07</td>
<td>4.10</td>
<td>4.31</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.95</td>
<td>2.95</td>
<td>2.99</td>
</tr>
<tr>
<td>Tyrosine&lt;</td>
<td>5.49</td>
<td>5.50</td>
<td>5.73</td>
</tr>
<tr>
<td>Lysine</td>
<td>10.5</td>
<td>10.1</td>
<td>10.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.05</td>
<td>4.00</td>
<td>4.05</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.02</td>
<td>2.96</td>
<td>2.84</td>
</tr>
<tr>
<td>1-Carboxymethylhistidine</td>
<td>0</td>
<td>0.99</td>
<td>0</td>
</tr>
<tr>
<td>3-Carboxymethylhistidine</td>
<td>0</td>
<td>0</td>
<td>1.01</td>
</tr>
<tr>
<td>Ammonia</td>
<td>18.6</td>
<td>21.7</td>
<td>17.1</td>
</tr>
</tbody>
</table>

* RCM denotes reduced, carboxymethylated.

<sup>a</sup> There are three isoleucine residues in ribonuclease, two in an isoleucylisoleucyl sequence which is incompletely hydrolyzed in 22 hours.

<sup>b</sup> Not corrected for decomposition during hydrolysis.

<sup>c</sup> Corrected for 0.1 residue of the decomposition products of carboxymethylmethionine sulfoxide salt (1, 14).

middle curve of Fig. 2 corresponded to RCM-Tryp 16<sup>a</sup>, residues 105 to 124. Based on the carboxymethylhistidine content, the yield was 35%. Upon analysis, the materials obtained from both of the new peaks in the bottom curve of Fig. 2 were found to include a carboxymethylhistidine-containing peptide corresponding to RCM-Tryp 4, residues 11 to 31. The peptide in the first peak is probably the pyridoxinecarboxylic acid analogue of the one in the second (cf. Smyth et al. (15)). Based on the carboxymethylhistidine content, the yields of modified RCM-Tryp 4 in these two peaks were 35% and 30%, respectively. Since none of these peptides was pure, further work was required to isolate purified derivatives.

<sup>a</sup> The nomenclature used for the peptides is the same as that of Hirs et al. (13, 17), except that the prefix RCM is employed to denote that the peptides have been derived from reduced, carboxymethylated ribonuclease or its derivatives.
FIG. 2. Comparison of chromatographic separation of peptides obtained after an 8-hour tryptic hydrolysis of reduced carboxymethylated ribonuclease A (top curve), reduced, carboxymethylated 1-carboxymethylhistidine-119 ribonuclease (center curve), and reduced, carboxymethylated 3-carboxymethylhistidine-12-ribonuclease (bottom curve). The 15-cm column of Amberlite IR-120 on the amino acid analyzer was used. To avoid confusion tracings of the curves obtained from Channel 1 only of the analyzer are shown here. Load, peptides from 5 mg of protein; flow rate, 30 ml per hour; temperature, 50°. The arrows denote the positions of peptides containing a residue of carboxymethylhistidine.

In the procedure which proved to be preferable for the isolation of carboxymethylated RCM-Tryp 16, 31 mg of the major alkylated product (cf. Fig. 1) were hydrolyzed with trypsin as before. The mixture of peptides was adjusted to pH 3 with N HCl and desalted on a column (2 X 5 cm) of Dowex 50-X2 in 0.2 M ammonium formate. The column was washed with 100 ml of 0.2 M ammonium formate, 20 ml of water, and then 30 ml of m NH4OH. The NH4OH fraction was taken to dryness by rotary evaporation after the addition of 5 mg of Na2CO3. The residue dissolved readily in 1 ml of water, and the solution was chromatographed on a column (0.9 X 15 cm) of IRC-50 in the hydrogen form, equilibrated with water. Elution was carried out at 5 to 10 ml per hour with a linear gradient extending from water to glacial acetic acid over a volume of 100 ml. This procedure was suggested by the previous observation (10) that large peptides—the reduced, carboxymethylated glyceyl and phenylalanyl chains of insulin—could be separated from one another easily by chromatography on the acid form of IRC-50 with 50% acetic acid as eluent. It had also been found that all of the smaller tryptic peptides from ribonuclease were eluted by 50% acetic acid. The effluent fractions were analyzed by measurement of absorbancy at 280 mp.

To isolate the carboxymethylhistidine-containing peptide from the minor alkylated product (Fig. 1, last peak), a portion of the 8-hour tryptic hydrolysate corresponding to 23 mg of protein was brought to pH 2.7 with N HCl and chromatographed on the 15-cm IR-120 column with the elution sequence shown in Fig. 2. The effluent was collected in a fraction collector. Resolution at the higher load was not as good as that shown in Fig. 2. Fractions corresponding roughly to the two indicated by the arrows over the bottom curve on Fig. 2 were pooled and stored at 4°. Crystals formed in the second fraction. Upon analysis they were found to contain 3-carboxymethylhistidine and to consist of a mixture of RCM-Tryp 2 and the carboxymethylhistidine analogue of RCM-Tryp 4 in a ratio of 2:1. The yield of the modified RCM-Tryp 4, based on the 3-carboxymethylhistidine content, was 21%. The large peptides in the mother liquor were isolated by passage through a column (0.9 X 50 cm) of Sephadex G-25 with 50% acetic acid as eluent. Absorbancy at 280 mp was used for detection of the peaks. The fractions corresponding to 6, 7, and 8 effluent ml were pooled and, upon amino acid analysis, yielded the data given in Table II.

**Synthesis of l- and S-Carboxymethylhistidine and Dicarboxymethylhistidine**—These derivatives were prepared for characterization and reference by alkylating α-N-acetyl-L-histidine (California Corporation for Biochemical Research) with iodooacetic acid, followed by acid hydrolysis and separation of the products on a column of IR-120 (cf. (1)).

Acetylhistidine (160 mg) was dissolved in 14 ml of water, and
TABLE II

Amino acid composition of tryptic peptides containing carboxymethylhistidine

The composition of each peptide is expressed as molar ratios of the constituent amino acids. Each column represents the results of a single analysis of a 22-hour hydrolysate.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>As isolated</th>
<th>After Edman degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino acid</td>
<td>From RCM-1-carboxymethylhistidine-119-KNaO₄</td>
<td>From RCM-3-carboxymethylhistidine-125-KNaO₄</td>
</tr>
<tr>
<td></td>
<td>(residues 103 to 142)</td>
<td>(residues 11 to 31)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>2.07</td>
<td>2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.08</td>
<td>1</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.46</td>
<td>1</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.05</td>
<td>2</td>
</tr>
<tr>
<td>Valine</td>
<td>3.80</td>
<td>4</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.03</td>
<td>0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.24</td>
<td>1</td>
</tr>
<tr>
<td>Serine</td>
<td>0.55</td>
<td>1</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.86</td>
<td>0</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.88</td>
<td>3</td>
</tr>
<tr>
<td>8-Carboxymethylleucines</td>
<td>1.01</td>
<td>1</td>
</tr>
<tr>
<td>Proline</td>
<td>2.04</td>
<td>2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.00</td>
<td>1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.08</td>
<td>1</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.05</td>
<td>0</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.06</td>
<td>1</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.06</td>
<td>1</td>
</tr>
<tr>
<td>1-Carboxymethylhistidine</td>
<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>3-Carboxymethylhistidine</td>
<td>0.04</td>
<td>1</td>
</tr>
</tbody>
</table>

The abbreviation is the same used in Table I.

Values for amino acids present to less than 0.01% residue are omitted.

These values are corrected for the presence of 10% RCM-Tryp 2, which has the composition, Asx, Glx, Gly, Ileu, Ser, Trp, Met, CMCys, Tyr, Arg.

The isoleucylisoleucyl sequence is not completely hydrolyzed in 22 hours.

Not corrected for decomposition occurring during hydrolysis.

Includes 0.22 residue of carboxymethylhomocysteine derived from the carboxymethylmethionine sulfonium salt (1, 14).

to the solution were added 540 mg of iodoacetic acid dissolved in 7 ml of 0.03 N NaOH. The mixture was brought to pH 8.1 by the addition of 2.6 ml of 2 N NaOH and the volume was adjusted to about 45 ml. After the addition of 225 mg of solid NaHCO₃, the solution was heated on a steam bath under reflux for 3 hours. To remove the acetyl group by hydrolysis, 45 ml of concentrated HCl and 135 ml of 2 N HCl were added, and the mixture was boiled under reflux for 5 hours. The HCl was removed by rotary evaporation, and 30 ml of water and 15 ml of buffer at pH 3.25 were added to bring the solution to pH 1.6. Chromatographic analysis of an aliquot gave the curve shown in Fig. 3. The main portion of the hydrolysate was then chromatographed in two portions on a column (4 X 16 cm) of IR-120 with a 0.2 N sodium citrate buffer at pH 3.25. The effluent was collected in 125-ml fractions, and 100-μl samples from alternate fractions were analyzed by the ninhydrin method. The column was regenerated by washing with 0.2 N NaOH and equilibration with buffer at pH 3.25 after each use.

The fractions constituting each of the three peaks were combined, and each sample was desalted (19) on a column (4 X 12 cm) of Dowex 2-X10 (200 to 400 mesh, fine mesh) before removal. Before the column, the resin in the chloride form was cycled once through the hydroxide form by successive washings with 2 liters of 2 N NaOH at room temperature, 1 liter of hot 2 N NaOH, water, approximately 1 liter of 4 N HCl, and water until neutral. After the column was poured, the resin was converted to the hydroxide form by passage of 2 liters of hot 2 N NaOH at a rate of about 500 ml per hour. The NaOH solution, which was prepared from 50% NaOH and boiled distilled water to minimize the CO₂ concentration, was at 60°C when added to the column. The column was then washed with water until the effluent was neutral. A fraction containing one of the histidine derivatives (volume of 100 to 300 ml) was added to the column; the column was washed with 1 liter of water and then with 2 N acetic acid. The effluent was collected in 20-ml fractions, and the histidine derivatives were located by ninhydrin analysis of 100-μl portions withdrawn from alternate fractions. The column was regenerated with 1 liter of 4 N HCl, water, and hot 2 N NaOH before reuse.

Each of the three products was isolated in essentially the same fashion. The desalted fractions containing the derivative were pooled and the acetic acid was removed by rotary evaporation. The residue was dissolved in about 3 ml of water and desalted once again on a column (0.9 x 4 cm) of Dowex 2-X10 by the same procedure used in the large scale desalting. After removal of the acetic acid, the products were dissolved in a small amount (1 x 4 ml) of hot water, and about 3 volumes of hot ethanol were added. The mixture was left at 4°C overnight; the crystals formed were filtered off, washed with ethanol, and dried in a vacuum desiccator over CaCl₂ and KOH before analysis. All three of the derivatives contained water of hydration which could not be removed completely even upon prolonged drying.

The yield of recrystallized dicarboxymethylhistidine from the fastest moving peak was 140 mg.

C₁₉H₁₇O₆N₄·H₂O (289)
Calculated: C 41.52, H 5.23, N 14.53, H₂O 6.30
Found: C 41.71, H 5.27, N 14.73, H₂O 5.23

The water loss of 5.23% was only about 80% of theory even though the substance was dried for 26 hours over P₂O₅ in a
high vacuum at 100°. The substance contained no ash. Analysis of the dried material for carbon and hydrogen gave values which were within 0.3% and 0.2% of those to be expected if about 80% of 1 mole of water had been lost.

The yield of 1 (or 3)-carboxymethylhistidine from the slowest moving peak was 140 mg.

\[ C_{6}H_{12}N_{2}O_{7} \cdot H_{2}O \ (231) \]

Calculated: C 41.56, H 5.67, N 18.18, H₂O 8.50

Found: C 41.54, H 5.85, N 18.28, H₂O 6.26

As with the dicarboxymethyl derivative, prolonged drying under the same conditions removed only about 75% of the theoretical quantity of water of crystallization. Analysis of the dried material was in accord with this figure. The substance was devoid of ash.

The yield of 1 (or 3)-carboxymethylhistidine from the central peak was 16 mg. The material contained 1.05% ash. It was dried over P₂O₅ at room temperature before analysis. The values are corrected for ash.

\[ C_{6}H_{12}N_{2}O_{4} \cdot \frac{1}{2} H_{2}O \]

Calculated: C 43.31, H 5.43, N 18.95

Found: C 43.55, H 5.24, N 18.40

Because of the scarcity of material, further purification was not attempted, but the substance, together with the other derivatives, was subjected to infrared spectroscopy, the results of which are presented in an appendix to this paper.

The color constant with ninhydrin for dicarboxymethylhistidine was 20.9 and that of the purest preparation of carboxymethylhistidine was 26.9 on an amino acid analyzer for which the neighboring serine and glycine color constants were 27.2 and 26.9, respectively. It was assumed that both carboxymethylhistidines have the same color constant.

RESULTS

Characterization of Carboxymethylhistidines—The derivative which gave the elementary analysis for a dicarboxymethyl compound was not examined in further detail; it can be expected to contain a betaine structure.

From the elementary composition of the other two compounds, it was concluded that both were monocarboxymethyl derivatives. One is 1-carboxymethylhistidine (α-amino-β-(1-carboxymethyl-5-imidazole)propionic acid), the other, 3-carboxymethylhistidine (α-amino-β-(1-carboxymethyl-4-imidazole)propionic acid) (see formulas).

From an inspection of molecular models, it was clear that in acetyl-L-histidine the 1-position is markedly sterically hindered both by the carboxyl group and by the α-acetyl amino group.

The 3-isomer would, therefore, be expected to be formed in the preponderant amount. Since the slowest moving peak at 290 ml was always far larger than the one at 195 ml (cf. Fig. 3), it was tentatively assumed to arise from the 3-isomer. This assignment is confirmed by the comparison, described in an appendix to this paper, of the infrared spectra of the two carboxymethylhistidines with the spectra of the authentic 1- and 3-methyl derivatives prepared by Tallan, Stein, and Moore (20).

In an attempt to increase the relative quantity of the 1-isomer, various conditions of alkylation were examined briefly. Under conditions similar to those employed for the extensive alkylation of ribonuclease (8.5 mM acetylhistidine, 150 mM iodoacetate, pH 5.5, 25°), less than 1% of the acetylhistidine had been transformed in 21 hours. To obtain a greater total yield of products, a higher pH, temperature, and concentration of reactants were required. The amount of the 3-isomer was always at least 3 times that of the 1-isomer, however. As the extent of alkylation increased, the relative percentage of the 1-isomer decreased, whereas that of the dicarboxymethyl derivative increased. Apparently the alkylation of the unhindered 3-position of the 1-carboxymethyl isomer proceeds almost as rapidly as the initial alkylation at the 1-position, whereas a second alkylation at the 1-position of the 3-carboxymethyl isomer is slow. This behavior also is to be expected from an examination of molecular models.

Histidine itself is alkylated more rapidly than N-acetylhistidine at pH 5.5 and 25°. After 21 hours, 5% of the amino acid had reacted. The ratio of the 3-carboxymethyl to the 1-carboxymethyl isomer was about 2:1. When poly-L-histidine was alkylated at pH 5.5, the two monocarboxymethyl isomers were formed in about equal amounts. Alkylation thus may be a useful means of studying the conformation of histidine residues in complex peptides, such as this polymer.

Characterization of Two Carboxymethyl Derivatives of Ribonuclease—The derivative formed in smaller amount in the experiments of Gundlach et al. (1) was not examined in detail at that time. The extent to which this compound is formed is shown by a chromatographic analysis of the mixture present after 2 hours of reaction with the alkylating agent (see Fig. 1). The results of amino acid analyses of the isolated derivatives (after reduction and carboxymethylation) are given in Fig. 4. The lower curve demonstrates that the minor product from the alkylation contains 3-carboxymethylhistidine, whereas it is the 1-isomer which is present in the major product (middle curve). Each purified derivative of ribonuclease is free of the other isomer of carboxymethylhistidine. The data in Table I show the presence of 1.0 residue of alkylated histidine per molecule of ribonuclease.

Gundlach et al. (1) were not able to detect enzymatic activity in that portion of the effluent which contained the minor component. The activities of the purified preparations obtained in the present study were compared with cyclic cytidylic acid as substrate. The 1-carboxymethylhistidine derivative was inactive (less than 1% of the activity of ribonuclease A); the 3-carboxymethyl derivative showed 7% activity. The significance of this observation is considered in the following paper (11).

When tested against nucleic acid, the 3-carboxymethyl derivative showed no proton release. Approximately 5% of the activity of ribonuclease A would have been detected.

Position of 1-Carboxymethyl- and 3-Carboxymethylhistidine Residues in Sequence—The results shown in Fig. 2 permit a comparison of the peptide patterns obtained from tryptic hy-
Allcylation of Histidines at Active Site of Ribonuclease

FIG. 4. Amino acid analyses of acid hydrolysates of A, reduced, carboxymethylated ribonuclease A; B, reduced, carboxymethylated 1-carboxymethylhistidine-119-ribonuclease; C, reduced, carboxymethylated 3-carboxymethylhistidine-12-ribonuclease. All analyses were carried out on the amino acid analyzer (9).

drolysates of reduced, carboxymethylated ribonuclease A and of the two reduced, carboxymethylated carboxymethylhistidine derivatives. Amino acid analyses of the fractions constituting the new peak of the middle curve (Fig. 2) suggested that the 1-carboxymethylhistidine residue occurred in a peptide obtained in over 35% yield which was analogous to the peptide O-Tryp 16, isolated by Hirs et al. (13, 17) (residues 105 to 124). The appearance of the new peak was not accompanied by the disappearance of another peak that could be attributed to unmodified RCM-Tryp 16, which is insoluble under these particular conditions of elution. The isolation of the modified RCM-Tryp 16 in excellent yield by chromatography of the peptide mixture on IRC-50 gave a pure peptide, as evidenced by the analyses given in Table II. The composition is that expected for a peptide comprising the carboxyl-terminal 20 amino acid residues of ribonuclease (13, 17). There are two histidine residues in this portion of the molecule, one at position 119, one at position 105. The latter is the amino-terminal residue in the peptide isolated, and therefore the substance was submitted to one stage of the Edman degradation by the procedure of Konigsberg and Hill (18) (cf. (15)). AR can be seen from the data in Column 5 of Table II, the residue of histidine was removed in over 60% yield, whereas the quantity of the 1-carboxymethylhistidine was virtually unaffected. It is clear, therefore, that nitrogen 1 of the histidine residue at position 119 was alkylated in the formation of the major inactive product.

This result is in harmony with the finding of Barnard and W. D. Stein (2, 3), who used different methods to examine the reaction mixture obtained by the alkylation of ribonuclease with C14-labeled bromoacetic acid.

The 3-carboxymethylhistidine residue in the minor product (Fig. 1) was found in two separate portions of the effluent. The fractions containing 3-carboxymethylhistidine in an over-all yield of 65% are designated by the two arrows in the lower curve of Fig. 2. A key feature of the amino acid analyses is that all of these fractions were devoid of proline, valine, phenylalanine, and unsubstituted histidine. The only peptide that could yield a carboxymethylhistidine-containing peptide devoid of these residues is RCM-Tryp 4 (cf. (13, 17)), representing residues 11 to 31. Carboxymethylhistidine-containing peptides that might arise from other parts of the molecule by the action of trypsin or chymotrypsin all would contain one or more of the amino acid residues mentioned. The rapid rate of movement of part of RCM-Tryp 4 probably indicates that the amino-terminal glutamine residue had undergone cyclization to form a residue of pyrrolidonecarboxylic acid (cf. (15)).

From the amino acid composition of the effluent fractions, it was clear that the desired peptide, the modified RCM-Tryp 4, was contaminated with about an equal quantity of RCM-Tryp 2. It was difficult to resolve this mixture. The purest material remained in the mother liquor after a crystalline mixture had separated from the fractions under the second arrow in the lower curve of Fig. 2. As indicated in Table II, this material, obtained in 6% yield, was composed of 87% of the 3-carboxymethylhistidine analogue of RCM-Tryp 4 and 13% of RCM-Tryp 2. Mixtures of these peptides in other proportions were also obtained (cf. "Experimental Procedure").

The peptide which contains the 3-carboxymethylhistidine residue is analogous to the peptide O-Tryp 4 characterized by Hirs et al. (13), which is now known to contain a histidine residue...
at position 12 (Smyth et al. (15)). It is this residue, therefore, that has been alkylated on nitrogen 3 of its imidazole ring.

**DISCUSSION**

The data presented in this communication demonstrate that when ribonuclease A is alkylated by iodoacetic acid at pH 5.5, two inactive products are formed, 1-carboxymethylhistidine-119-ribonuclease and 3-carboxymethylhistidine-12-ribonuclease. Since there is formed approximately 8 times as much of the histidine-119- as the histidine-12- derivative, it is possible that the latter might arise by alkylation of a minor component still present in chromatographically homogeneous ribonuclease A. Such a hypothetical material could have a different amino acid sequence from ribonuclease A, could have the same sequence but a different arrangement of disulfide bonds, or could be a conformational isomer. It might arise from a different strain of cow, or from transformations during isolation. The following experimental findings bear on these possibilities. The minor product has the same amino acid composition as ribonuclease A (with one residue of histidine replaced by one residue of 3-carboxymethylhistidine); it yields a similar pattern of peptides when a tryptic hydrolysate of the oxidized or reduced protein is fractionated (with, of course, the exception of the position of the peptide containing the alkylated histidine); and a peptide has been isolated which has the same amino acid composition (except for histidine) as the one comprising residues 11 to 31 from oxidized ribonuclease A. Heating ribonuclease A in 0.35 M NaCl at 65° for 10 minutes, which would be expected to alter the ratio of conformational isomers, did not alter the course of the subsequent alkylation reaction. Finally, the small amount of heterogeneity found in preparations of ribonuclease A by the use of sulfoethyl-Sephadex (cf. (4)) was not responsible for the two products; the major fraction obtained from such columns yielded the usual ratio of derivatives upon alkylation. A sample of ribonuclease A isolated by Dr. C. H. W. Hirs from the pancreatic juice obtained from a single cow yielded the two alkylation products. The weight of evidence, therefore, supports the conclusion that the two derivatives are formed from a single molecular species, ribonuclease A.

Further studies of the factors which influence the alkylation reaction, of the properties of the derivatives, and of the implications of these findings for the structure and conformation of the active site of the enzyme are presented in the following paper.

**APPENDIX: SPECTRAL CHARACTERIZATION OF THE CARBOXYMETHYLHISTIDINES**

Herman Jaffe

From The Rockefeller Institute, New York 21, New York

In order to decide whether the positions assigned to the carboxymethyl groups in the isomeric carboxymethylhistidines could be confirmed spectrosopically, infrared spectra of the two derivatives and of the known 1- and 3-methylhistidines (20) were determined in KBr with the aid of a model 137 Infracord spectrophotometer equipped with sodium chloride optics.

As can be seen in Fig. 1, a well defined spectrum was obtained for each compound. There is a distinctive difference in the spectral patterns of the 1- and 3-methylhistidines in the range from 11 μ to 13 μ, which is denoted in the figure by the stippled bands. A corresponding difference is seen in the band profiles of the carboxymethylhistidines. This comparison confirms the structures assigned to the 1- and 3-carboxymethylhistidines. Further confirmation is provided by the significant absorption band at 12 μ, which is indicated by the open double headed arrow, and is common to the 1-carboxymethyl and 1-methyl derivatives but is absent in the spectra of their respective isomers. This band appears to be analogous to the characteristic out-of-plane

SUMMARY

When ribonuclease A is alkylated by iodoacetic acid at pH 5.5 in the absence of polyvalent anions, two chromatographically distinct monocarboxymethyl derivatives are formed. The yield of one is approximately 8 times that of the other. The activities of the isolated derivatives were compared against cyclic cytidylic acid as substrate. Relative to ribonuclease A, the major product showed undetectable activity (less than 1%); the minor product showed 7% activity. Against RNA, the minor product showed less than 5% of the activity of ribonuclease A. Upon acid hydrolysis, both products were found to contain a single carboxymethylhistidine residue. The chromatographic behavior of the carboxymethylhistidines obtained from the two derivatives was different, however, indicating that substitution had occurred on nitrogen 1 of the imidazole ring in one case, and on nitrogen 3 in the other. The two isomers were synthesized by alkylation of ε-N-acetylhistidine, followed by acid hydrolysis. The products were isolated on ion exchange columns and were characterized by elemental analysis and infrared spectroscopy. Comparison of the chromatographic behavior of the synthetic carboxymethylhistidine derivatives with those obtained from the alkylated ribonucleases proved that the major product contained one residue of 1-carboxymethylhistidine, and the minor product one residue of 3-carboxymethylhistidine. Each of the ribonuclease derivatives was reduced with mercaptoethanol and alkylated with iodoacetic acid to transform all of the half-cystine residues to residues of carboxymethylcysteine. The reduced, carboxymethylated derivatives were hydrolyzed with trypsin, and the peptides from each protein that contained the carboxymethylhistidine residues were isolated by ion exchange chromatography and subjected to amino acid analysis. In this way it was demonstrated that in the major inactive product alkylation had occurred at the histidine residue occupying position 119 to yield 1-carboxymethylhistidine-119-ribonuclease. In a similar fashion, the minor inactive product was identified as 3-carboxymethylhistidine-12-ribonuclease.

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The spectra of both carboxymethylhistidines show a doubling of the (N+) band at about 4.5 μ which suggests that these compounds may exist as di-zwitterions. The unsubstituted ring nitrogen would presumably assume the second positive charge and give rise to the band at 5 μ.

A satisfactory spectrum of dicarboxymethylhistidine could not be obtained. Apparently, the overload of carboxyl groups caused interactions which obliterated practically all of the useful structural details of the spectrum.

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