The Abnormal Carboxyl Groups of Ribonuclease. II. Positions in the Amino Acid Sequence*

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ABSTRACT: Eight of the eleven carboxyl groups of ribonuclease A can be methylated. This methylated protein possesses many physicochemical properties which are similar to those of the native molecule, and it has been concluded that some or all of the free carboxyl groups in this derivative are the "buried" carboxyl groups of ribonuclease. In this communication the carboxyl groups which did not undergo esterification are identified. Proteolytic digestions (with trypsin and chymotrypsin), performed on the oxidized methylated derivative followed by peptide analyses, indicated that aspartic acid-14, aspartic acid-38, and aspartic acid-83 were present as the free carboxyl groups. With the identification of these carboxyls it is now possible to propose specific tyrosine-carboxylate interactions in the ribonuclease A molecule.

In the preceding communication (Broomfield *et al.*, 1965), the preparation and characterization of a methylated ribonuclease derivative was described. This derivative was shown to be methylated at eight of the eleven carboxyl groups known to be present in ribonuclease A. In addition, this derivative was shown to possess many of the characteristics of the native molecule. It was noted that the derivative underwent temperature and *p*H transitions which were quite similar to those of ribonuclease A. It was also possible to demonstrate that the derivative contained abnormally ionizable tyrosyl residues, and that two of the free carboxyl groups titrated with an abnormally low *pK*. Since other experimental information from this laboratory (Hermans and Scheraga, 1961a,b) has suggested the presence of tyrosine-carboxylate interactions in ribonuclease, and since Cha and Scheraga (1963) and Donovan (1963) have identified the location of two and one, respectively, of the buried tyrosines, it was of interest to identify the carboxyl groups which were present in the methylated ribonuclease derivative.

It is important to note that, despite the fact that the ribonuclease was methylated in alcoholic HCl (conditions under which the conformation of the native molecule is different from that at neutral *p*H), a partially methylated derivative was obtained which, like native ribonuclease, has abnormal tyrosyl residues which are near carboxyl groups. Hence, it is reasonable to assume that the partially methylated derivative refolds to the native conformation when dissolved in water at neutral *p*H.[1]

This paper reports the positions in the amino acid sequence of ribonuclease of the three carboxyl groups which did not undergo esterification. The identification was based on a comparison of the peptide elution patterns obtained from proteolytic digestions of the performic acid–oxidized methylated ribonuclease derivative to the elution patterns obtained from oxidized ribonuclease A digests. The peptides which contained unaltered carboxyl groups appeared in elution positions identical to those observed for these peptides from the oxidized ribonuclease A digests. On the other hand, because of the alteration in charge, the peptides which contained methylated carboxyl groups were not found at positions noted for these peptides when an oxidized ribonuclease A digest was chromatographed. In certain cases we were able to locate the altered peptides at new positions in the elution patterns.

Experimental

Materials. The preparation of methylated ribonuclease was identical to that previously described (Broomfield *et al.*, 1965). These methylated samples were routinely titrated at 25° and shown to contain three carboxyl groups. Salt-free trypsin and crystallized salt-free chymotrypsin were obtained from the Worthington Biochemical Corp. Carboxymethylcellulose was purchased from the Brown Co. Analytical grade Dowex 50-X2 resin

* From the Department of Chemistry, Cornell University, Ithaca, N.Y. *Received November 6, 1964.* This is paper XVI in the series, "Structural Studies of Ribonuclease." This work was supported by a research grant (AI-01473) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, U.S. Public Health Service, and by a research grant (GB-2238) from the National Science Foundation. Part of this work was presented before the Division of Biological Chemistry at the 140th meeting of the American Chemical Society, Chicago, September 1961.

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[1] Of course, the possibility always exists that, in the refolded partially methylated protein, the abnormal tyrosyl residues could be near different carboxyl groups than in the native molecule.

which was purchased from Bio-Rad Laboratories was prepared according to the procedure of Hirs *et al.* (1956a). L-Glutamic acid γ-methyl ester was a Mann Research Laboratories, Inc., product. All other reagents were either reagent grade or the best grade available.

Chromatography of the Methylated Ribonuclease. Analytical chromatography was performed on 0.9 × 20–cm columns of carboxymethylcellulose in conjunction with a Technicon Autoanalyzer. The columns were equilibrated with 0.015 M sodium phosphate buffer, *p*H 6.40, which was 0.2 M in sodium chloride. The chromatograms were developed by employing a two-stage Varigrad; the first stage contained 100 ml of 0.015 M phosphate buffer, *p*H 6.47 (0.2 M in sodium chloride) and the second stage contained 100 ml of *p*H 6.47 phosphate buffer (0.015 M; 0.5 M in sodium chloride). Preparative chromatography (100 mg of protein) was carried out on 1.8 × 30–cm columns of carboxymethylcellulose. The development of the chromatograms was similar to the analytical procedure with the exception that 300 ml was employed in each Varigrad stage. Following chromatography (flow rate of 20 ml/hour) the protein fractions were pooled, exhaustively dialyzed against HCl at *p*H 3.0, and then lyophilized.

Performic Acid Oxidation. Oxidations of the proteins were carried out at −10° as described by Hirs (1956).

Chromatography of the Oxidized Methylated Protein. Since the oxidized protein was extremely insoluble in salt solutions of low concentration at neutral *p*H, chromatography was performed at *p*H 5.0 where this complication did not arise. Analytical chromatography was carried out on 0.9 × 20–cm carboxymethylcellulose columns which were equilibrated with *p*H 5.0, 0.005 M acetate buffer. The chromatograms were developed by employing two-stage Varigrads in conjunction with a Technicon Autoanalyzer; the first stage contained 75 ml of 0.005 M acetate buffer, *p*H 5.0, while the second contained 75 ml of 0.005 M acetate buffer, *p*H 5.0, which was 0.4 M in sodium chloride.

The Problem of Ester Hydrolysis. THE LOSSEN REARRANGEMENT EXPERIMENTS. The Lossen rearrangement was carried out on the purified and oxidized components according to the procedure described by Broomfield *et al.* (1965).

EXPERIMENTS WITH L-GLUTAMIC ACID γ-METHYL ESTER. Solutions of L-glutamic acid γ-methyl ester (0.1 mM of amino acid per ml) which contained equivalent amounts of aspartic acid and serine were allowed to stand (a) 12 hours at *p*H 7.0 and (b) 12 hours at *p*H 3.0. The solutions were maintained at the desired *p*H by the addition of 0.1 N sodium hydroxide with the aid of a Radiometer *p*H-stat. After this time, the solutions were diluted to 2 μM of each amino acid per ml and then chromatographed on the amino acid column. Performic acid oxidation as described by Hirs (1956) was carried out on another sample of L-glutamic acid γ-methyl ester (1 mM). Again this sample contained equivalent amounts of aspartic acid and serine. After dilution to an appropriate amino acid concentration, the sample was chromatographed on the amino acid column. In other experiments, 50 mg of the ester was dissolved in 10 ml of water and the *p*H was raised to 6.8. To this solution was added 0.2 mg of trypsin (or chymotrypsin) in 1 ml of water. The temperature was maintained at 25° by employing a jacketed titration vessel, and the *p*H of the solution was kept at 6.8 by the addition of 0.1 N sodium hydroxide with the aid of the *p*H-stat. At the end of 6 hours, the solution was diluted to 2 μM of amino acid per ml and then 1 ml was chromatographed on the amino acid column.

Tryptic Digestions. Approximately 30–40 mg of the oxidized methylated derivative was dissolved in water (5 ml). The *p*H was adjusted to 6.8 by the addition of 0.1 N sodium hydroxide and then 0.2 mg of trypsin dissolved in 1 ml of water was added. The temperature was maintained at 25° and the *p*H of the solution was kept at 6.8 by the addition of 0.1 N sodium hydroxide with the aid of the *p*H-stat. Following reaction for 20 hours, the precipitate which had started to form after 0.5 hour's reaction time was centrifuged, washed well with water, and then dissolved by suspension in water and lowering of the *p*H to 3.0. This solution which will be termed O-T-precipitate was stored in the frozen state until needed. The supernatant tryptic digest was brought to *p*H 3.0 by the addition of 0.1 N HCl and then was immediately chromatographed on a 0.9 × 150–cm column of Dowex 50-X2.

Tryptic digestion of oxidized ribonuclease A was performed in a manner which was similar to that described for the methylated derivative. However, in this case no precipitate was observed. Therefore, following a 20-hour reaction time, the *p*H was lowered to 3.0 and then the solution was chromatographed on the Dowex 50-X2 column.

Chymotryptic Digestion. Chymotryptic digestions of oxidized ribonuclease A, of oxidized methylated ribonuclease, and of O-T-9 (the nomenclature is that of Hirs *et al.*, 1956a) were performed according to the method previously described for the tryptic digestion of oxidized ribonuclease A.

Chymotryptic digestions of O-T-precipitate were accomplished by first raising the *p*H of the O-T-precipitate solution to 6.0 and then by adding 0.2 mg of chymotrypsin in water (1 ml). No appreciable precipitation occurred during the chymotryptic hydrolysis. The reaction temperature was maintained at 25° and the *p*H was held at 6.0 for 3 hours. After this time, the *p*H was raised to 6.8 and then an additional 0.2 mg of chymotrypsin dissolved in 1 ml of water was added. Hydrolysis was allowed to proceed for an additional 18 hours, after which time the *p*H of the solution was lowered to 3.0 and then the hydrolysate was chromatographed on the Dowex 50-X2 column.

Separation of the Peptides on Dowex 50-X2. The procedure for the chromatography of the tryptic and chymotryptic digests of oxidized ribonuclease A and oxidized methylated ribonuclease was similar to that outlined by Hirs *et al.* (1956a,b) and Cha and Scheraga (1963). For the tryptic digests a better separation between O-T-2 and O-T-4 was observed when the gradient was begun after 300 ml of the starting buffer had been

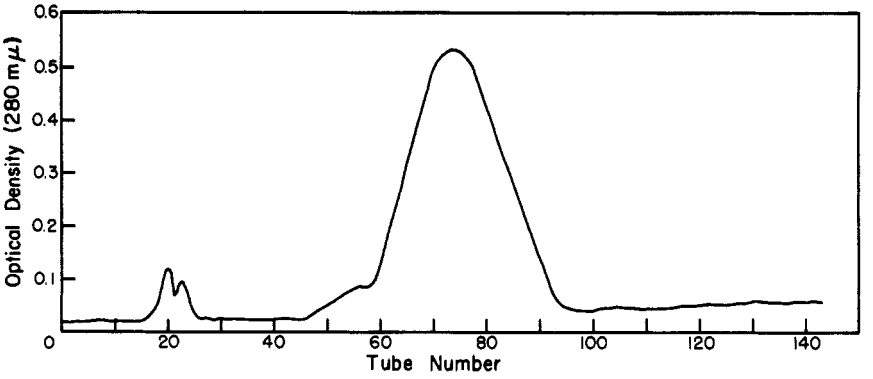

FIGURE 1: Chromatography of methylated ribonuclease on a 1.8 × 30–cm carboxymethylcellulose column. A two-stage gradient was employed; the first stage contained 300 ml of 0.015 M phosphate buffer, *p*H 6.47 (0.2 M in sodium chloride), and the second stage contained 300 ml of 0.015 M phosphate buffer, *p*H 6.47 (0.5 M in sodium chloride). The effluent (flow rate of 20 ml/hour) was collected in 4-ml fractions and the optical density was read at 280 mμ.

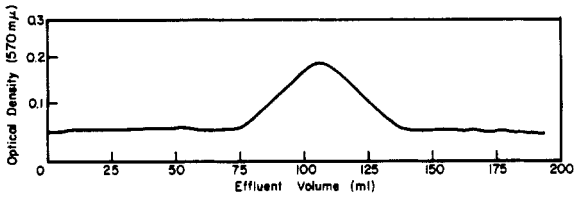

FIGURE 2: Analytical chromatography of the methylated ribonuclease on a 0.9 × 20–cm carboxymethylcellulose column following the preparative chromatographic procedure. A two-stage gradient was used. The first stage contained 100 ml of 0.015 M phosphate buffer, *p*H 6.47 (0.2 M in sodium chloride). The second stage contained 100 ml of phosphate buffer, 0.015 M, *p*H 6.47 (0.5 M in sodium chloride). The ninhydrin color value of the effluent (flow rate of 20 ml/hour) was obtained with the aid of a Technicon Autoanalyzer.

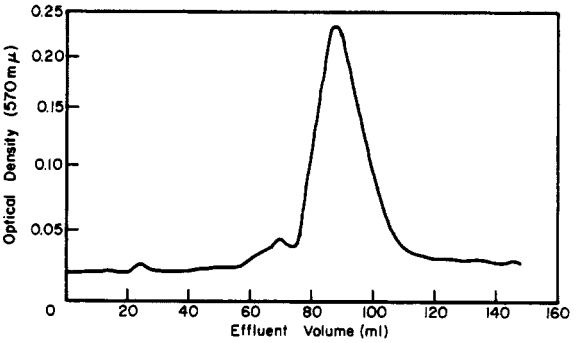

FIGURE 3: Analytical chromatography of the oxidized methylated protein on a 0.9 × 20–cm carboxymethylcellulose column. A two-stage gradient was employed; the first stage contained 75 ml of 0.005 M acetate buffer, *p*H 5.0, while the second contained 75 ml of 0.005 M acetate buffer, *p*H 5.0, which was 0.4 M in sodium chloride. The ninhydrin color value of the effluent, which had a flow rate of 20 ml/hour, was obtained with the aid of a Technicon Autoanalyzer.

eluted in place of 200 ml. The techniques for chromatographing the chymotryptic digests of O-T-9 and O-T-precipitate were those employed by Hirs (1960).

Amino Acid Analyses. Analyses were performed with the Technicon amino acid analyzer. Hydrolyses were carried out with 6 N HCl in sealed, evacuated ampules for 22 hours at 116°. The amino acid content of ribonuclease and its derivatives was calculated by assuming that twelve residues of alanine were present per mole of protein. The amino acid composition of each peptide hydrolysate was calculated by assuming that an appropriate amino acid was present in the theoretical amount and then employing this value to determine the number of residues for the other amino acids. The correction factors of Gundlach *et al.* (1959) and Rupley and Scheraga (1963) were employed for those amino acids which undergo decomposition during hydrolysis.

Results

Chromatography of Methylated Ribonuclease. Ideally, chromatography of the methylated protein should be performed at high *p*H. Such a condition would overcome the high positive charge on the protein and would undoubtedly result in clear resolutions between derivatives which differed only slightly in charge. However, because of the danger of effecting saponification under these conditions, it was deemed advisable to perform the chromatography at or around neutrality. Figure 1 illustrates a preparative chromatogram of the methylated protein (100 mg) from a 1.8 × 30–cm carboxymethylcellulose column. Although the main protein component appeared as a broad band, the peak was quite symmetrical. The center portion (tubes 67–82) of this zone was pooled and dialyzed against HCl at

TABLE I: Aspartic Acid, Glutamic Acid, and Valine[a] Content of the Methylated Proteins following the Lossen Rearrangement (moles of amino acid per mole of protein).

Amino Acid	Theory	Ribonuclease A Control	Methylated Ribonuclease	Methylated Ribonuclease (upon purification)	Oxidized Methylated Ribonuclease
Aspartic acid	15	14.66	12.81	13.25	13.17
Glutamic acid	12	11.79	7.52	7.59	7.71
Valine	9	8.88	7.81	8.15	8.24
2,3-Diaminopropionic acid			1.91	2.12	1.86
2,4-Diaminobutyric acid			2.86	1.81	2.02

[a] Valine is the C-terminal amino acid in ribonuclease.

*p*H 3.0. Subsequent lyophilization yielded 60 mg. Figure 2 illustrates an analytical chromatogram of the pooled protein from tubes 67–82 of Figure 1. Again, this protein band was extremely broad but symmetrical. A sharper band could be obtained by employing a steeper salt gradient (0.2–0.8 M in sodium chloride); this chromatogram appeared to contain a single component.

Figure 3 illustrates an analytical chromatogram of the oxidized methylated protein. In this case, the main protein band was eluted over a narrower salt range. Since the major component comprised at least 95% of the total protein, purification by preparative chromatography was not performed.

The Problem of Ester Hydrolysis. LOSSEN REARRANGEMENT EXPERIMENTS. Table I illustrates the yields of aspartic acid, glutamic acid, and valine obtained from the methylated protein following the purification and oxidation steps. Included in this table are the results observed for the methylated derivative (prior to purification) and the control ribonuclease experiment. The latter results were taken from the previous paper (Broomfield *et al.*, 1965). Within experimental error, these results indicated that the purified and oxidized proteins were esterified at one valine residue, five glutamic acid residues, and two aspartic acid residues. Since these results are similar to the methylated derivative (prior to purification), it appears that these steps have not caused any significant ester hydrolysis.

EXPERIMENTS WITH L-GLUTAMIC ACID γ-METHYL ESTER. This compound was eluted from the amino acid column just prior to the elution position of glycine; no additional ninhydrin-positive components were noted in this sample. In every case, chromatography of the L-glutamic acid γ-methyl ester samples, treated as reported in the experimental section, indicated that the ester was recovered in quantitative yields. In addition, these chromatograms were deficient in glutamic acid. Therefore, hydrolysis of the ester did not occur when this compound was allowed to stand at *p*H 7.0 or 3.0 for extended reaction times. It is also noteworthy that hydrolysis did not occur when the ester was subjected to the oxidation procedure described by Hirs (1956), nor when trypsin and chymotrypsin were allowed to stand in contact with this compound.

TABLE II: Amino Acid Analyses of O-T-6 and O-T-10 Obtained from a Tryptic Digest of Oxidized Methylated Ribonuclease (moles of amino acid per mole of peptide).

	O-T-6		O-T-10	
Amino Acid	Theory	Experimental	Theory	Experimental
Cysteic acid				0.13
Methionine sulfone				
Aspartic acid				
Threonine	1	1.00	1	1.10
Serine	2	2.17		0.20
Glutamic acid	1	1.12	1	1.13
Proline				
Glycine	1	1.00[a]		
Alanine			3	3.00[a]
Valine		0.21		0.14
Isoleucine				
Leucine				
Tyrosine				
Phenylalanine				
Lysine	1	0.96	2	1.69
Histidine				
Arginine				

[a] Assumed as reference.

Tryptic Digestions. Figure 4 illustrates the peptide elution pattern obtained from tryptic digests of oxidized ribonuclease A and the oxidized methylated derivative. The elution positions and the yields of the individual peptides observed for the ribonuclease A digest (Figure 4a) compared favorably with the yields and the elution positions previously described by Hirs *et al.* (1956a).

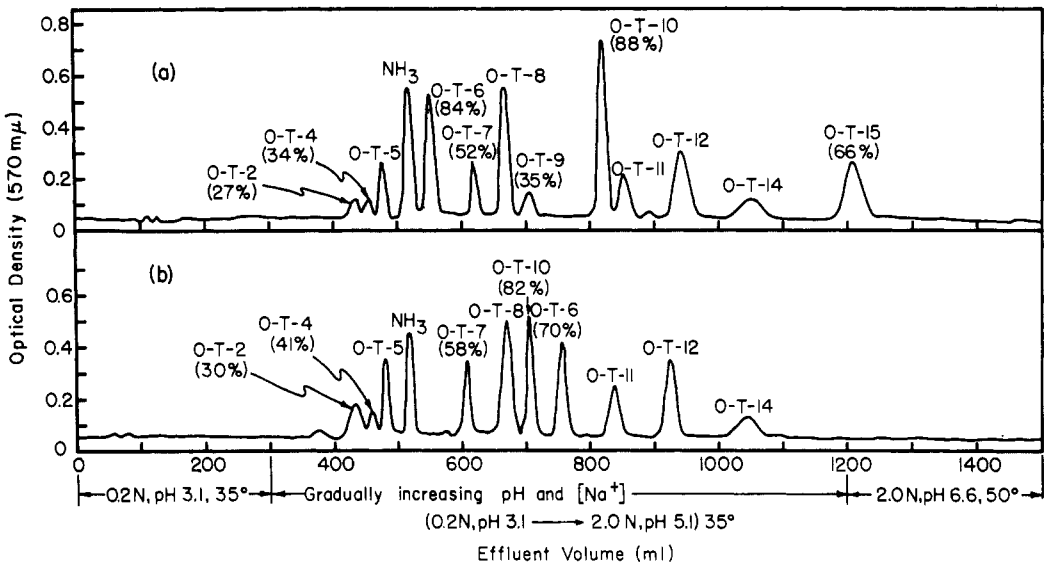

FIGURE 4: Chromatography of peptides from a 20-hour tryptic digest of oxidized protein on a 0.9 × 150–cm column of Dowex 50-X2. The effluent was collected in 2-ml fractions and the ninhydrin color values were obtained with the aid of a Technicon Autoanalyzer. The percentages in parentheses represent the yields of the pertinent peptides. (a) Ribonuclease A; (b) methylated ribonuclease.

On the other hand, a number of differences were noted in the peptide pattern obtained from the oxidized methylated ribonuclease digest (Figure 4b). Peptides O-T-6, O-T-9, O-T-10, and O-T-15[2] were not observed at their normal elution positions. In addition, O-T-6 and O-T-10 were found at new positions. Although these peptides appeared at new positions, their yields compared favorably with the amounts found in the oxidized ribonuclease A digest. The amino acid contents obtained for these altered peptides are reported in Table II.

The yields and the elution positions of the remaining peptides obtained from the oxidized methylated derivative agreed with the amounts and the elution positions observed for these peptides in the ribonuclease A digest. Brief comment should be made concerning the analyses of O-T-2 and O-T-4. Although the peptide zone ascribed to O-T-2 was predominantly this peptide (70% of the zone could be ascribed to O-T-2), an accurate amino acid analysis could not be obtained when the entire zone was pooled and a portion was analyzed. On the other hand, the front half of this zone yielded an analysis for O-T-2 which was relatively free of contaminating amino acids in both the unmethylated and methylated samples. The latter half of this zone contained, in addition to O-T-2, peptide(s) which could not be accurately determined. This observation may reflect the presence of small amounts of chymotrypsin in the trypsin preparation. Chymotryptic digestion, especially on the larger tryptic peptides, could produce peptides which appear near this zone. This observation has been noted by Hirs (1962). The peptide zone which has been termed O-T-4 contained an additional component which analyzed as O-T-2-C-5 (Hirs, 1960). This component has been found by Hirs (1962) to appear just prior to O-T-4 in tryptic digests of oxidized ribonuclease. It is noteworthy that O-T-2-C-5 consists of residues 80–85. Since O-T-2, O-T-4, and O-T-7 are of primary concern, the analyses of these peptides are reported in Table III.

The peptide elution pattern obtained from the oxidized methylated derivative showed no presence of O-T-9 or O-T-15. Elution with 2 N sodium acetate, *p*H 6.6, also proved ineffective in producing these peptides. Peptide O-T-16 which was not observed in the oxidized ribonuclease A digest was also absent in the oxidized methylated ribonuclease digest.

Studies on O-T-Precipitate. Amino acid analyses performed on this precipitate are reported in Table IV. The table shows the results from two different preparations. These analyses indicated that the precipitate was comprised of O-T-9 and O-T-16 and that the yields were approximately 30 and 55%, respectively.

Attempts to carry out the Lossen rearrangement on this precipitate were unsuccessful. This fact is probably due to the insolubility of O-T-precipitate at neutral *p*H (hydroxamic acid formation from the esters being carried out at neutrality), and exchange is undoubtedly much slower under these conditions.

Since O-T-9 and O-T-16 contain a total of five carboxyl groups in an oxidized ribonuclease A digest, it was of great interest to determine the state of these carboxyls in the O-T-precipitate. Therefore, it was decided to perform a chymotryptic hydrolysis of this

[2] The terminology employed in this communication is that of Hirs *et al.* (1956a) for the tryptic peptides and of Hirs *et al.* (1956b) and Hirs (1960) for the chymotryptic peptides.

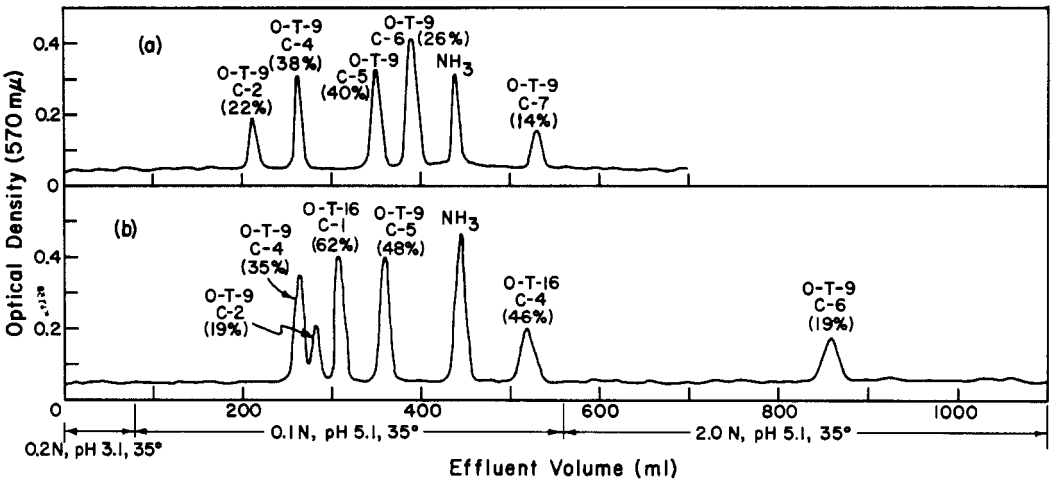

FIGURE 5: Chromatography of peptides from chymotryptic digests of O-T-9 (from unmethylated ribonuclease) and O-T-precipitate (from methylated ribonuclease). The percentages in parentheses represent the yields of each peptide. (a) O-T-9; (b) O-T-precipitate.

TABLE III: Amino Acid Analyses of O-T-2, O-T-4, and O-T-7 Obtained from a Tryptic Digest of Oxidized Methylated Ribonuclease (moles of amino acid per mole of peptide).

	O-T-2[a]		O-T-4[b]		O-T-7	
Amino Acid	Theory	Observed	Theory	Observed	Theory	Observed
Cysteic acid	2	1.79	1	1.04		0.12
Methionine sulfone	1	1.07	3	2.68		0.14
Aspartic acid	3	3.19	3	3.05	1	1.30
Threonine	3	2.81	1	1.10		0.11
Serine	3	3.38	6	5.80		0.34
Glutamic acid	2	2.32	2	1.96		
Proline						
Glycine	1	1.00[c]				
Alanine		0.29	2	2.00[c]		
Valine		0.26		0.20		
Isoleucine	1	0.83				
Leucine						
Tyrosine	2	1.88	1	0.82		
Phenylalanine						
Lysine			1	0.80		0.14
Histidine			1	0.85		0.11
Arginine	1	0.88			1	1.00[c]

[a] The analysis was obtained from the front half of the peptide zone. [b] The analysis has been compensated for the presence of O-T-2-C-5. [c] Assumed as reference.

precipitate. Figure 5 illustrates the peptide elution pattern obtained from a chymotryptic digest of the O-T-precipitate from methylated ribonuclease. This figure also includes the elution pattern obtained from a chymotryptic digest of O-T-9 (Figure 5a) from unmethylated ribonuclease; this pattern was similar to that previously reported by Hirs (1960). On the other hand, the pattern obtained from the chymotryptic digestion of the O-T-precipitate (Figure 5b) indicated that three of the peptides to be expected from O-T-9 (O-T-9-C-2, O-T-9-C-6, and O-T-9-C-7) were not present in their normal elution positions. Peptides O-T-9-C-2 and O-T-9-C-6 were found in new positions and their amino acid analyses are reported in Table V.

The elution patterns obtained from the chymotryptic digest of the O-T-precipitate also contained peptides arising from O-T-16. Two such peptides were noted (O-T-16-C-1 and O-T-16-C-4) and their amino acid

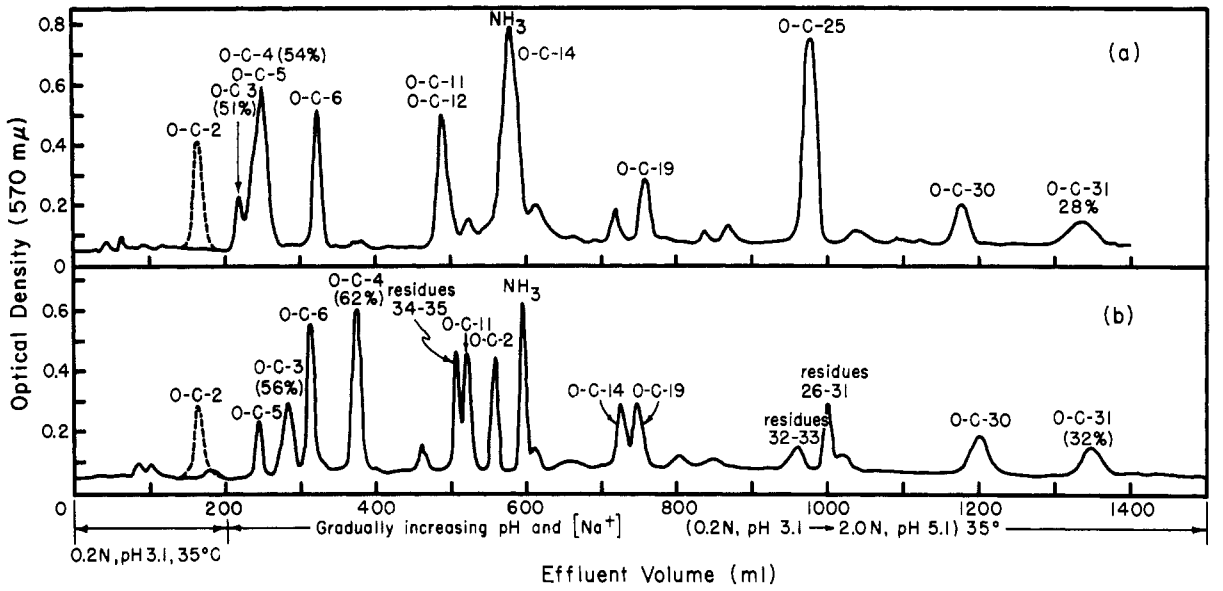

FIGURE 6: Chromatography of peptides from a 20-hour chymotryptic digest of oxidized protein. The chromatographic conditions are the same as in Figure 4 except for the volume of buffer change. The percentages in parentheses represent the yields of the pertinent peptides. (a) Ribonuclease A; (b) methylated ribonuclease.

TABLE IV: Amino Acid Analyses of O-T-Precipitate (moles of amino acid per mole of peptide).

	Theory		Observed (Expt 1)		Observed (Expt 2)	
Amino Acid	O-T-9	O-T-16	O-T-9	O-T-16	O-T-9	O-T-16
Cysteic acid	2	1	2.00[a]	0.90	2.00[a]	0.96
Methionine sulfone			0.11		0.07	
Aspartic acid	2	2	2.00[a]	2.29	2.00[a]	2.02
Threonine	1		1.25		1.33	
Serine	2	1	2.00[a]	1.17	2.00[a]	1.13
Glutamic acid	3	1	3.00[a]	1.14	3.00[a]	0.80
Proline	1	2	1.00[a]	1.90	1.00[a]	1.95
Glycine		1		1.00[a]		1.00[a]
Alanine	2	2	2.00[a]	1.90	2.00[a]	1.66
Valine	4	4	4.00[a]	3.20	4.00[a]	3.31
Isoleucine[b]		2		1.11		1.16
Leucine	1		1.00[a]		1.00[a]	
Tyrosine		1		0.90		0.98
Phenylalanine	1	1	1.00[a]	0.78	1.00[a]	0.92
Lysine	2		2.27		1.82	
Histidine	1	2	1.00[a]	1.79	1.00[a]	1.86
Arginine			0.17		0.18	

[a] O-T-9 was assumed to be present in the theoretical amount, corresponding to 1.00 residue of leucine (since O-T-16 does not contain leucine). The data for O-T-16 were then obtained by assuming that 1.00 residue of glycine was present in the hydrolysate. [b] Isoleucine is not completely liberated from O-T-16 in a 22-hour acid hydrolysis.

compositions are also reported in Table V. The third peptide (O-T-16-C-5) was not found in the chromatogram.

Chymotryptic Digestions. Figure 6 illustrates the peptide elution pattern obtained from chymotryptic digests of oxidized ribonuclease A and oxidized methylated ribonuclease. The peptide elution pattern obtained from oxidized ribonuclease A was similar to

TABLE V: Amino Acid Analyses of the Peptides Obtained from a Chymotryptic Digest of the O-T-Precipitate (moles of amino acid per mole of peptide).

	O-T-9-C-2		O-T-9-C-6		O-T-16-C-1		O-T-16-C-4	
Amino Acid	Theory	Observed	Theory	Observed	Theory	Observed	Theory	Observed
Cysteic acid		0.18		0.11			1	0.95
Methionine sulfone								
Aspartic acid	1	1.19	1	1.22	1	1.04	1	1.07
Threonine				0.14				0.16
Serine			1	0.89	1	1.01		0.11
Glutamic acid	1	1.28	2	2.26		0.36	1	1.06
Proline							1	0.88
Glycine		0.11				0.27	1	1.00[a]
Alanine	1	1.00[a]	1	1.00[a]	1	1.00[a]	1	1.02
Valine	1	1.06	2	2.15	1	1.05	1	0.92
Isoleucine[b]							2	0.76
Leucine			1	0.76				
Tyrosine							1	0.62
Phenylalanine								
Lysine								
Histidine			1	0.87			1	0.83
Arginine								

[a] Assumed as reference. [b] Isoleucine is not totally liberated in a 22-hour acid hydrolysis.

that reported by Hirs *et al.* (1956b).[3] On the other hand, a number of differences were noted in the oxidized methylated ribonuclease digest. The major points of interest are the elution positions of O-C-3 and O-C-4; these peptides were not observed at their normal elution positions. O-C-3 (residues 106–115) was eluted after O-C-5 while O-C-4 (residues 121–124) appeared after O-C-6. The yields of these peptides compared favorably with the yields obtained from an oxidized ribonuclease A digest. The amino acid analyses of O-C-3 and O-C-4 are reported in Table VI.

Other differences were noted. O-C-14 (residues 80–97, containing Asp-83 and Glu-86) was observed in a new position just prior to O-C-19. In addition, we have not observed zones corresponding to O-C-12 and O-C-25. Each of these peptides in an oxidized ribonuclease A digest contains at least one carboxyl group. Peptide O-C-27 (residues 26–35), which does not contain a carboxyl group, was also absent. However, a peptide zone at 500 effluent ml analyzed as Asp-Leu (residues 34–35) while another zone at 950 effluent ml analyzed as Ser-Arg (residues 32–33). In addition, a zone at 1000 effluent ml was composed of Cys,Met_2,Asp,Glu, Lys (residues 26–31). Thus it appears that O-C-27 has been cleaved at the Lys-Ser (31–32) and the Arg-Asp(NH_2) (33–34) bonds, perhaps owing to the presence of a small amount of trypsin in the chymotrypsin preparation.

It is also interesting that O-C-2 (residues 74–76) appeared at two positions. One zone (a ninhydrin-negative zone) appeared at 200 effluent ml and was detected by basic hydrolysis. This position was identical to that reported by Hirs *et al.* (1956b). Another zone at 550 effluent ml also analyzed as O-C-2.

O-C-31 (residues 36–46) was observed in a position identical to that reported for the oxidized ribonuclease A digest. The amino acid analysis of this peptide is also reported in Table VI. It is noteworthy that the yields of this peptide in both the oxidized ribonuclease A digest and in the oxidized methylated ribonuclease digest were not as large as the yields reported by Hirs *et al.* (1956b). This may be due to tryptic digestion of the Lys-Asp (37–38) and/or the Arg-Cys (39–40) bond.

Discussion

In studies which attempt to identify the "buried"[4] carboxyl groups of a protein, the ideal experimental design would be one in which the protein derivative is formed in an aqueous medium. However, our attempts to perform such reactions have, up to the present time, proved unsuccessful. The use of a water-soluble carbodiimide has been investigated. It was

[3] A number of peaks reported by Hirs *et al.* (1956b) were not identified in the present work. Most of these peaks were obtained in small yield and were not characterized by these workers.

[4] The term "buried" is used to define those carboxyl groups which are believed to be involved in interactions with other groups in the protein.

TABLE VI: Amino Acid Analyses of O-C-3, O-C-4, and O-C-31 Obtained from a Chymotryptic Digest of Oxidized Methylated Ribonuclease (moles of amino acid per mole of peptide).

Amino Acid	O-C-3		O-C-4		O-C-31	
	Theory	Observed	Theory	Observed	Theory	Observed
Cysteic acid	1	1.17			1	1.22
Methionine sulfone						
Aspartic acid	1	1.00[a]	1	1.16	2	2.00[a]
Threonine		0.21			2	1.78
Serine			1	1.06		0.23
Glutamic acid	1	1.25		0.31		0.18
Proline	1	1.00			1	0.89
Glycine	1	0.94				
Alanine	1	1.11	1	1.00[a]		
Valine	1	1.02	1	1.10	1	1.13
Isoleucine[b]	2	1.11				
Leucine						
Tyrosine	1	0.87				
Phenylalanine					1	0.92
Lysine					2	1.86
Histidine						
Arginine					1	0.94

[a] Assumed as reference. [b] Isoleucine is not totally liberated in a 22-hour acid hydrolysis.

visualized that the carbodiimide would form activated protein carboxyl intermediates and then these activated intermediates could be converted to the more stable hydroxamic acids. Although the carboxyl groups of ribonuclease did apparently react with the carbodiimide, as determined by an uptake of acid, we have been unsuccessful in demonstrating that these activated carboxyl intermediates could be converted to the hydroxamic acid derivatives. The reaction between the carbodiimide and ribonuclease is under further investigation in our laboratory.

Esterification of the carboxyl groups in ribonuclease has been attempted by employing a water-soluble diazo compound. In this case, it was possible to demonstrate that esterification had occurred; however, the extent to which the reaction proceeded was quite limited. A more detailed communication on these observations is reported in the following paper (Riehm and Scheraga, 1965).

A ribonuclease derivative which was esterified at eight of the eleven carboxyls could be prepared from a methanolic HCl medium, as described by Broomfield *et al.* (1965). It is admitted that the medium in which this derivative was prepared is nonideal. Nevertheless, even though the derivative was prepared in methanolic HCl, it does dissolve in water at neutral *p*H, it does have three unmethylated carboxyl groups, it does have buried tyrosyl groups, and it does appear to have a conformation similar to that of the native molecule. Hence, despite its method of preparation, the resulting derivative justifiably may be assumed to possess the same tyrosyl-carboxyl interactions as the native one;[1] it was thus of interest to extend our studies to an identification of those carboxyl groups which had not been esterified. In view of the identification of two of the "buried" tyrosine residues by Cha and Scheraga (1963), such identifications could provide valuable information about the three-dimensional structure of the protein.

Reduction procedures whereby the esters in the methylated protein would be converted to the more stable alcohols were considered. However, there appears to be no satisfactory reducing procedure which will convert esters to alcohols without reducing peptide linkages or side-chain amide bonds. Therefore, it was decided to investigate the methylated derivative by studying the peptide elution patterns obtained from proteolytic digests of the oxidized protein. Such examinations undoubtedly depend on the stability of the ester linkages since hydrolysis at any point in the procedure would yield erroneous results. However, hydrolysis did not appear to be a source of difficulty in this work. Thus a protein derivative, which by titration was shown to be methylated at eight of the eleven carboxyl groups, was also shown to be lacking two aspartic acid residues, five glutamic acid residues, and one valine residue by the Lossen rearrangement experiment. In addition, the protein derivative was lacking these amino acid residues following the purification and oxidation steps. It is also noteworthy that the model compound L-glutamic acid γ-methyl ester was resistant to hydrolysis during the oxidation procedure of Hirs (1956). In addition, this compound was shown to be resistant to hydrolysis when it was allowed to stand at *p*H 7.0 or 3.0 for extended reaction times. These extremes of *p*H were the

extremes to which the methylated protein (and its resulting proteolytic digests) was subjected.

Another possibility for error is the problem of ester hydrolysis caused by the esterase action of trypsin and chymotrypsin. Again, this possibility does not appear to exist since these enzymes were found not to catalyze the hydrolysis of the model compound L-glutamic acid γ-methyl ester.

A further source of error in these studies could be *N,O*-acyl migrations occurring during the esterification procedure. These reactions involve the side-chain hydroxyls of serine and threonine and result in the formation of esters from peptide linkages with the release of the α-amino groups from the serine and threonine residues. Although most observations concerning such reactions have been noted under more rigorous experimental conditions, Chibnall and Rees (1953) have reported that insulin in 0.1 N methanolic HCl and at 25° underwent this migration between the Gly-Ser bond (residues 8–9 in the B chain). Therefore, it was of great importance to determine the extent (if any) to which this reaction occurred during the esterification of ribonuclease.

It is interesting to note that the titration curve (Broomfield *et al.*, 1965) of the methylated ribonuclease showed no abnormalities between *p*H 5 and 8. If *N,O*-acyl migration had occurred, then a certain fraction of the threonine and serine α-amino groups would be expected to titrate in this range. In addtiion, any rapid reversal over this range would also be noted since this reaction involves the release of hydrogen ions. It is also noteworthy that the Lossen rearrangement experiments showed no decrease in the serine and threonine content of the methylated derivative. Since this technique involves treating the protein with dinitrofluorobenzene, one might well expect to observe a decrease in these amino acids if *N,O*-acyl migration had occurred.

In addition, in every case except one, those peptides (from the oxidized derivative) which contained threonine and serine, and which were lacking free carboxyl groups, appeared in positions identical to the elution positions noted for these peptides when oxidized ribonuclease A digests were chromatographed. If *N,O*-acyl migration had occurred, the appearance of these amino groups would undoubtedly affect the elution position of the peptides. The single exception to these observations was the appearance of O-C-2 (residues 74–76; Glu-(NH_2)-Ser-Tyr) at two positions. O-C-2 is normally eluted at approximately 200 effluent ml as a ninhydrin-negative peptide and it was only at this position that the peptide was noted in the oxidized ribonuclease A digest. The failure to produce a positive ninhydrin test suggested to Hirs *et al.* (1956b) that this peptide contained an amino-terminal pyrrolidonecarboxylic acid, formed by the cyclization of an amino-terminal glutamine residue. The appearance of O-C-2 at an additional position in the oxidized methylated digest is probably due to a fraction of the peptide which did not undergo cyclization rather than the result of any *N,O*-acyl migration. It is also noteworthy that the tryptic digest of the oxidized methylated protein showed that O-T-2 (residues 67–85) was observed at only one position (the position normally ascribed to O-T-2).

Identification of Methylated Carboxyls. The method for determining the state of the eleven carboxyls (the free carboxyls of ribonuclease A) in the methylated ribonuclease derivative was based on the assumption that the methylated carboxyl groups would give rise to peptides (upon proteolytic digestion of the oxidized derivative) which would chromatograph differently than the peptides obtained from an oxidized ribonuclease A digest. Although the esterification of a particular carboxyl group in a certain peptide will not be observed in an amino acid analysis of the peptide, since acid hydrolysis will yield the corresponding acid from its ester, the alteration in charge upon the methylation of a carboxyl group should be sufficient to change the elution position of a peptide containing this altered carboxyl group. Thus, in the case of the tryptic digest of the oxidized methylated derivative it was noted that O-T-6, O-T-9, O-T-10, and O-T-15 were not found at their normal elution positions. Therefore, it is believed that the carboxyl groups in these peptides (Glu-86 in O-T-6, Glu-2 in O-T-10, and Glu-9 in O-T-15) were esterified in the methylated derivative. O-T-9 contains two carboxyl groups in the native molecule (Glu-49 and Asp-53) and it was not possible to state that *both* carboxyls had been esterified. Nevertheless, the absence of O-T-9 from its normal elution point indicates that at least one of the carboxyl groups had been esterified. It is also encouraging to note that these peptides were completely absent from their normal elution positions. Appreciable amounts of these peptides at their usual positions would have indicated that the methylated derivative was a randomly esterified component.

Further evidence that the carboxyl groups in O-T-6 and O-T-10 were methylated arises from the observation that these peptides were observed in new positions. Thus O-T-10 was eluted immediately after O-T-8 and O-T-6 appeared 10 ml after O-T-10. The esterification of a particular carboxyl group would be expected to increase by one unit the positive charge of a peptide containing this esterified carboxyl group. Therefore, it would be expected that the altered peptides would chromatograph at a slower rate on the Dowex 50-X2 peptide column. Such was the case with the altered O-T-6 peptide. On the other hand, the altered O-T-10 peptide chromatographed more rapidly than was observed for this peptide in the oxidized ribonuclease A digest. No plausible explanation can be given for this observation. It is noteworthy that this was the only altered peptide which chromatographed at a faster rate than did the identical peptides from an oxidized ribonuclease A digest. The peptide O-T-15 was not observed; eluting the column with 2 N, *p*H 6.6 acetate buffer failed to yield this peptide.

The elution positions for O-T-2, O-T-4, and O-T-7 obtained from the tryptic digest of the oxidized methylated derivative were identical to the positions observed for these peptides when a tryptic digest of oxidized ribonuclease A was chromatographed. In addition, we did not observe these peptides at other posi-

tions. It is also noteworthy that O-T-2-C-5 (residues 80–85) was observed in hydrolysates of O-T-4. This peptide, which arises from chymotryptic digestion of O-T-2 (the chymotrypsin was apparently an impurity in the trypsin preparation), was noted both in the oxidized ribonuclease A digest and the oxidized methylated derivative digest as an O-T-4 impurity. Therefore, it appears that the carboxyl groups in these peptides (Asp-83 in O-T-2 and in O-T-2-C-5, Asp-14 in O-T-4, and Asp-38 in O-T-7) were not esterified in the methylated derivative.

The tryptic experiments yielded information on the state of six of the eleven carboxyls in the methylated derivative. Glu-2, Glu-9, and Glu-86 were shown to be methylated while Asp-14, Asp-38, and Asp-83 appeared to be present as the free carboxyl groups. It is also evident that at least one of the two carboxyl groups in O-T-9 (Glu-49 and/or Asp-53) is esterified.

The five carboxyls which were not identified from the tryptic experiments are to be found in O-T-9 and O-T-16. It is noteworthy that these peptides precipitated from solution when a tryptic digest was carried out on the oxidized methylated derivative; if one were to assume that all the carboxyls in these peptides were esterified then the net charge on each peptide would be zero at neutral *p*H, accounting for the insolubility.

Chymotryptic digestion of the O-T-precipitate has yielded some knowledge about the state of these five carboxyl groups. Thus it is seen, in the case of the O-T-precipitate, that O-T-9-C-2 (residues 52–55, containing Asp-53), O-T-9-C-6 (residues 47–55, containing Glu-49 and Asp-53), and O-T-9-C-7 (residues 47–51, containing Glu-49) were not found in their normal elution positions. In addition, we have observed O-T-9-C-2 and O-T-9-C-6 at new positions. Therefore, it is believed that both Asp-53 and Glu-49 are esterified in the methylated protein. Although a comparison between chymotryptic digests of O-T-16 and O-T-precipitate was not made (O-T-16 was not found in a tryptic digest of the oxidized ribonuclease A), it is believed that at least one of the carboxyls in O-T-16-C-1 (residues 121–124, containing Asp-121 and Val-124) is esterified. It is known from peptic digestion studies of oxidized ribonuclease (Bailey *et al.* 1956) that the tetrapeptide O-P-2 (residues 121–124) is eluted at a faster rate than is O-P-3 (residues 52–55). Therefore, if the tetrapeptide O-T-16-C-1 (residues 121–124) had not been altered, one would expect it to chromatograph at a faster rate than the peptide containing residues 52–55. A visual comparison of Figure 5 indicates that this was not the case.

The peptide elution pattern obtained from the chymotryptic digest of the oxidized methylated protein indicated that Glu-111 was esterified. As was noted previously, peptide O-C-3 (residues 106–115, containing Glu-111) was not eluted at its normal elution position. In addition, this peptide was observed at a new position. It is also noteworthy that O-C-4 (residues 121–124) was not observed at its normal position. This observation again indicated that either Asp-121 or Val-124 (or both) was esterified.

It is believed that Val-124 was esterified in the methylated protein. As was noted previously, the Lossen rearrangement indicated that one valine residue was esterified in the methylated protein. The only possibility for the esterification of a valine carboxyl (in the ribonuclease molecule) is at Val-124.

These studies have not been able to determine the state of Asp-121. Nevertheless, in view of the fact that Asp-14, Asp-38, and Asp-83 were shown to be present as the free carboxyl groups, and since the methylated protein was shown to contain but three carboxyl groups (Broomfield *et al.*, 1965), it is believed that Asp-121 was esterified.

The results of these studies may be summarized as follows. Tryptic digests indicated that Glu-2, Glu-9, and Glu-86 were esterified while Asp-83, Asp-38, and Asp-14 were present as the free carboxyl groups. Chymotryptic digests of O-T-precipitate indicated that Asp-53 and Glu-49 were esterified. Chymotryptic digestion of the oxidized methylated protein suggested that Glu-111 was esterified and that Asp-121 or Val-124 (or both) were esterified. The Lossen rearrangement experiments showed that Val-124 was esterified. It is also noteworthy that the results of the Lossen rearrangement technique indicated that the three free carboxyl groups in the methylated derivative were aspartic acid residues. This observation was consistent with the subsequent findings that Asp-14, Asp-38, and Asp-83 were present as the free carboxyl groups.

Structural Implications. Since two of the "buried" tyrosine residues in ribonuclease A (tyrosines 25 and 97) have been identified by Cha and Scheraga (1963) it should be possible to pair these residues, in a reasonable manner, with two of the "buried" carboxyl groups. It is known that a disulfide linkage occurs between Cys-26 and Cys-84 (Spackman *et al.*, 1960). Therefore, it is concluded that Asp-83 and Tyr-25 could be involved in a tyrosine-carboxylate hydrogen bond. Such an interaction could explain the specific chymotryptic cleavages observed by Rupley and Scheraga (1963) upon taking ribonuclease through its temperature transition. These workers noted that primary cleavages occurred at the Tyr-25 and Cys-26 bond and also at the Met-Ser (79–80) bond. It was concluded that these portions of the peptide chain unfolded in the thermal transition, an unfolding which could occur upon breaking the Tyr-25 to Asp-83 hydrogen bond.

The choice for pairing the remaining "buried" tyrosyl residue (Tyr-97) with either Asp-14 or Asp-38 appears to favor Asp-38. The major reason for this assumption is the presence of a disulfide linkage between Cys-40 and Cys-95 (Spackman *et al.*, 1960). Therefore, interaction between Tyr-97 and Asp-38 is visualized as very probable. In addition, the primary chymotryptic cleavages at the Leu-Thr (35–36) bond and Tyr-Lys (97–98) bond observed by Rupley and Scheraga (1963) lend support to a Tyr-Asp (97–38) hydrogen bond. The pairings, as proposed, do not exclude an interaction between Asp-14 and either Tyr-25 or Tyr-97. However, Ooi and Scheraga (1964) have proposed that the N-terminal tail (residues 1–25) is related to the peptide Cys-84

to Lys-104 such that Glu(NH_2)-11 to Ala-20 reacts with the chain between Cys-84 and Cys-95. If this proposal is correct, then it appears to exclude an interaction between Asp-14 and either Tyr-25 or Tyr-97.

It is not possible, at this time, to pair Asp-14 with a tyrosine residue. However, with the identification of the third "buried" tyrosine, perhaps the remaining carboxyl group will also be paired in a reasonable fashion. Also, studies are being carried out in this laboratory, involving iodination and methylation of pepsin-inactivated ribonuclease (Anfinsen, 1956). Since pepsin-inactivated ribonuclease contains only one abnormal tyrosine (Bigelow and Ottesen, 1959), results of such experiments will enable us to pair the tyrosyl and carboxyl groups properly.

The conclusions presented in this communication are intended to add to the knowledge about the conformation of this protein in solution. Thus, the specific carboxyl-tyrosyl interactions proposed (Tyr-25 to Asp-83 and Tyr-97 to Asp-38) place restrictions on the manner in which the molecule could fold. These pairings may possibly be revised when our results on pepsin-inactivated ribonuclease are obtained. Other information on the structure of this protein is available and is summarized here. The arrangement of the disulfide bonds in ribonuclease (Spackman *et al.*, 1960) greatly restricts the conformation that this protein can assume in solution. In addition, Crestfield *et al.* (1963) postulate that histidine residues 12 and 119 are in close proximity (approximately 5 A apart) and form part of the active site. The data of Hirs (1962) and of Cooke *et al.* (1963) also suggest that Lys-41 is near these histidine residues. These conclusions are further supported by Saroff and Carroll (1962), who propose that there are two triplet-positive sites in ribonuclease; subsequently, M. Loeb and H. A. Saroff (private communication) proposed additional triplet-positive sites.

It is visualized that future experiments (such as the use of cross-linking reagents) will elucidate the structure of additional parts of the molecule. Nevertheless, it is recognized that a protein could exist in many conformations and that the results of such experiments would yield information on the most stable conformation of a certain portion of the molecule.

All of this information is being considered in current efforts at calculating protein structure with the aid of computer methods (Nemethy and Scheraga, 1965).

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