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Structural Studies of Ribonuclease. XXI. The Reaction between Ribonuclease and a Water-Soluble Carbodiimide*

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ABSTRACT: As a further attempt to modify the carboxyl groups of ribonuclease in an aqueous medium, we report that the reaction between this protein and a water-soluble carbodiimide (at pH 4.5) produces a number of ribonuclease derivatives. Although the exact nature of the reaction product is not known (it is probably either an acylurea or an imide), five derivatives (designated as B, D, F, G, and H) have been isolated and examined. It appears that each of these components differs from ribonuclease in the number of carboxyl groups which have been modified. Peptide analyses and titration data indicate that component B is a derivative which has been modified at one of the eleven carboxyl groups (Asp-53) of ribonuclease. Similar studies on component D indicate that this derivative possesses three modified carboxyl groups (Asp-53, Glu-49, and Glu-111). Component F appears to be a mixture of two derivatives; approximately half of this component is a ribonuclease derivative which has reacted at Asp-53, Glu-49, Glu-111, and Glu-9, while the remaining half has reacted at Asp-53, Glu-49, Glu-111, and Glu-86. Component G has been modified at five of the eleven carboxyl groups (Asp-53, Glu-49, Glu-111, Glu-9, and Glu-86) of the native molecule. In addition to these five carboxyl groups, component H has also been modified at Asp-38. Thermal transition studies and spectrophotometric titration data indicate that each derivative possesses a somewhat looser structure than does ribonuclease. However, components B, D, F, and G appear to contain three abnormal tyrosyl residues, as does the native molecule. On the other hand, component H contains only *two* abnormal tyrosyl residues. Since this derivative differs from component G only in that reaction has occurred at Asp-38, it is concluded that Asp-38 could be involved in a specific tyrosyl–carboxylate interaction believed to be present in ribonuclease.

It has been inferred, from ultraviolet difference spectral data and optical rotation data at low pH, that one or more of the three abnormal tyrosyl residues in ribonuclease (Shugar, 1952; Tanford *et al.*, 1955) are involved in specific tyrosyl–carboxylate interactions (Hermans and Scheraga, 1961). Hermans and Scheraga suggested that these interactions exist in nonpolar regions of the molecule. The existence of specific tyrosyl–carboxylate interactions is further supported by the fact that, in order to fit the experimental potentiometric titration curve with a theoretical curve, it is necessary to assume that some of the carboxyl groups possess a lower pK^0 (intrinsic ionization constant) than do the remainder.

Since the sequence of amino acids in ribonuclease is known, the identification of the abnormal tyrosyl residues as well as the identification of the "buried"[1] carboxyl residues would add valuable information about the conformation of this protein in solution.

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[1] The term "buried" refers to those carboxyl groups which are believed to be involved in specific tyrosyl–carboxylate interactions.

Cha and Scheraga (1963) and Donovan (1963) have reported the identification of two and one, respectively, of the abnormally titrating tyrosyl residues, and work is currently in progress in this laboratory to identify the third. In addition, Fujioka and Scheraga (1965) have identified the one abnormal tyrosyl residue in pepsin-inactivated ribonuclease.

Broomfield *et al.* (1965) reported on the preparation and characterization of a methylated ribonuclease in which eight of the eleven carboxyl groups of the native molecule were esterified. Subsequently, Riehm *et al.* (1965) identified the three free carboxyl groups as Asp-14, Asp-38, and Asp-83, and it was concluded that some or all of these carboxyl groups could be the "buried" carboxyl groups of ribonuclease. However, even though the methylated derivative possessed physicochemical properties which were similar to those of the native molecule, it was pointed out that, since the derivative was prepared from methanolic HCl (a denaturing medium), the partially methylated protein may have refolded (upon solution in water) in a manner such that the abnormal tyrosyl residues were near different carboxyl groups than in the native molecule. In order to circumvent this difficulty, studies were made of possible protein carboxyl reactions which could be carried out in aqueous media. The use of a diazo compound was investigated (Riehm and Scheraga, 1965), and it was noted that, although ribonuclease appears to contain at least one extremely reactive carboxyl group (Asp-53), the extent of esterification was quite limited. The fact that the diazo compound was not a satisfactory reagent for blocking all the "exposed"[2] carboxyl groups in ribonuclease was probably due (to a large degree) to the hydrolysis of the reagent. As a further attempt to obtain protein carboxyl reactions in an aqueous medium, we now wish to report our observations on the reaction between ribonuclease A and a water-soluble carbodiimide.

Sheehan and Hlavka (1957) published the first studies of the reaction between a protein and a water-soluble carbodiimide, stating that the reaction produced interchain cross-linking in gelatin. This cross-linking was presumably due to amide formation occurring between side-chain carboxyl and amino groups. In addition, Goodfriend *et al.* (1964) reported that a water-soluble carbodiimide produced coupling of bradykinin and rabbit serum albumin. On the other hand, by carrying out the reaction between a protein and a water-soluble carbodiimide at pH values (pH 4.0) where coupling by nucleophilic groups (such as lysine) on the protein should be nearly or totally inhibited, Franzblau *et al.* (1963) have noted that the reaction between gelatin and a water-soluble carbodiimide (in the presence of hydroxylamine) yielded hydroxamic acid derivatives of side-chain aspartic acid and glutamic acid residues. These workers also presented evidence which indicated that a large amount of acylurea and/or imide formation had occurred. Thus, it seemed logical that a water-soluble carbodiimide in the presence of a protein, at low pH, would lead to protein derivatives which arise from carboxyl group reaction.

We have used the WSC[3] to modify the carboxyl groups of ribonuclease. Although the exact nature of the reaction product is not known (see Discussion), it is probably either an acylurea (IV) or an imide (V), formed by means of the unstable acylisourea intermediate (III) (Khorana, 1955), according to either of the reaction pathways shown in Scheme I. In this reaction scheme compound I is the WSC and II is a portion of a polypeptide chain with an aspartic acid side chain. Both IV and V would yield aspartic acid upon acid hydrolysis.

The reaction at pH 4.5 (a pH which should normally limit the reaction to carboxyl groups) between ribonuclease and the WSC yielded a number of chromatographically distinguishable components. Five derivatives (denoted as B, D, F, G, and H) have been isolated and examined. It appears that each component differs from ribonuclease only in the number of modified carboxyl groups. This fact is supported by titration, hydrolyses, and peptide chromatography data. Amide formation, either inter- or intramolecular, appears to be negligible or nonexistent. Each of the components possessed enzymatic activity; however, the activity ranged from a few per cent to 100% depending on the derivative. All components underwent thermal transitions in the acid range which were similar to ribonuclease, and all derivatives contained abnormally titratable tyrosyl residues. However one component (component H) appeared from spectrophotometric titrations and thermal transition studies to contain only *two* abnormal tyrosyl residues. Since it was possible to demonstrate by peptide analyses that component H differed from component G in that Asp-38 was modified, it is concluded that Asp-38 could be involved in a specific tyrosyl–carboxylate interaction in ribonuclease.

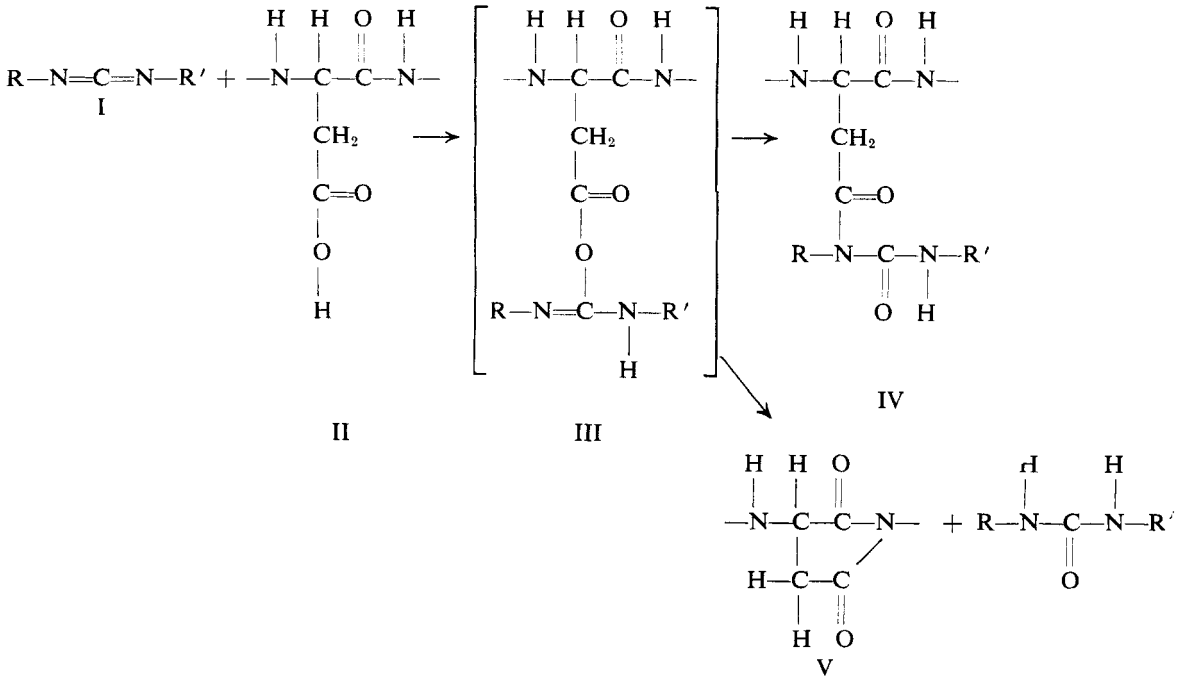
Experimental Section

Materials. Five-times-crystallized ribonuclease (Lot R23 B-55) was purchased from the Sigma Chemical Co. The ribonuclease A fraction was prepared by methods previously described (Rupley and Scheraga, 1963). The WSC was an Aldrich Chemical Corp., Inc., product. Ninhydrin and hydrindantin were purchased from Pierce Chemical Co. Amberlite MB-1 resin was obtained from the Rohm and Haas Co. All other reagents were either reagent grade or the best grade available.

Methods. PREPARATION OF DERIVATIVES. In the initial stages of this study the reaction was carried out by employing dilute protein solutions. Approximately 250

[2] The term "exposed" refers to those carboxyl groups which are believed not to be involved in specific tyrosyl–carboxylate interactions.

[3] This abbreviation will be used for the specific water-soluble carbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, employed in this investigation. This particular compound was selected because of its commercial availability.



SCHEME I

mg of ribonuclease A was dissolved in 500 ml of water at room temperature and the solution was brought to pH 4.5 by the addition of 0.1 N HCl. To the stirred solution was added 250 mg of WSC, and the pH was maintained at 4.5 by the use of 0.1 N HCl and a Radiometer Titrator (TTT1) pH-Stat. The reaction was allowed to proceed at room temperature for 6 hr and then the solution was dialyzed in the cold (2°) against six changes of water which were 0.001 N in HCl. After lyophilization the protein (215 mg) was stored at 2° until needed. In another experiment, additional quantities of WSC (250 mg each) were added after 6- and 12-hr reaction time, respectively. Upon stirring at room temperature for a total of 24 hr, the reaction mixture was dialyzed in a manner identical with that previously described and then lyophilized to yield 228 mg of product.

Higher protein concentrations (approximately 5 mg/ml) were utilized in other experiments. Approximately 250 mg of ribonuclease A was dissolved in 50 ml of water and the solution was placed in a jacketed titration vessel. The temperature of the solution was maintained at 2° by circulating water through the vessel from an external bath. The pH of the solution was lowered to 4.5 by the addition of 1 N HCl, and then 250 mg of WSC was added. The pH of the solution was maintained at 4.5 by the addition of 1 N HCl from the pH-Stat. Additional quantities (250 mg) of WSC were added every 6 hr (three additions a day) until the desired number of additions was obtained. The protein solution was dialyzed against six changes of water which was 0.001 N in HCl and then lyophilized. In this manner, products were obtained after three, six, and nine additions of WSC.

REACTION BETWEEN RIBONUCLEASE AND THE WSC AT pH 9.5. Approximately 50 mg of ribonuclease was dissolved in 20 ml of water and the pH was raised to 9.5 by the addition of 0.1 N NaOH. To this solution was added 50 mg of the WSC, and the reaction was allowed to proceed at pH 9.5 and at room temperature for 12 hr. At the end of this time the pH was lowered to 3.0 and the reaction mixture was dialyzed against six changes of water which was 0.001 N in HCl. Subsequent lyophilization yielded 40 mg of protein. A portion of this material was prepared for amino acid analysis.

CHROMATOGRAPHY. Preparative chromatography was carried out on 1.8 × 30 cm columns of Bio-Rex 70. The chromatograms were developed with the aid of a Technicon Autograd employed as a four-stage gradient. Since the pH and molarity of the buffers differed from run to run, the conditions for chromatographing the reaction products will be described in the following section. Analytical chromatography was performed on 0.9 × 30 cm columns of Bio-Rex 70 in conjunction with a Technicon Autograd and Autoanalyzer.

RIBONUCLEASE ACTIVITY. Activity of the derivatives was measured by the change in absorbancy at 300 mμ which occurs in the enzymic depolymerization of RNA in 0.1 M sodium acetate buffer at pH 5.0, as described by Kunitz (1946).

SPECTRAL STUDIES. Spectrophotometric titrations at 295 mμ were carried out with a Beckman DU spectrophotometer. The pH, which was measured with a Beckman Model GS pH meter, was changed by successive additions of 1 N KOH.

Ultraviolet difference spectral measurements were carried out with a Cary Model 14 recording spectrophotometer. The temperatures of the reference and sample cells were independently controlled. The difference spectrum (from 4 to 65°) was measured at 287 mμ by maintaining the sample cell at 4° and the reference cell at a temperature which was varied from 4

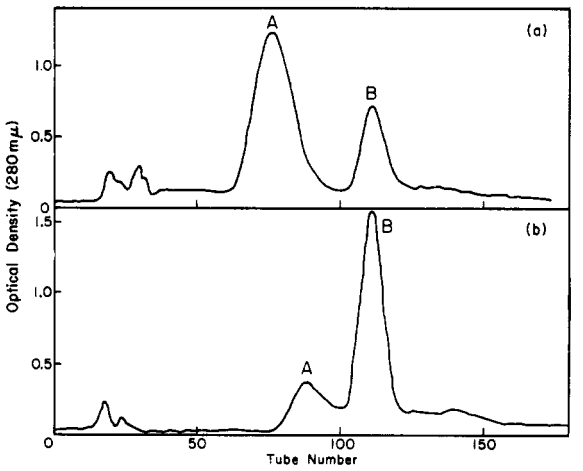


FIGURE 1: Chromatography of 200 mg of protein obtained from the reaction between the WSC and ribonuclease. Dilute protein solutions (0.5 mg/ml) were employed to obtain the reaction products. A four-stage gradient was employed the first three stages contained 200 ml of 0.15 M sodium phosphate buffer, pH 6.40, while the fourth stage contained 200 ml of 1 M sodium phosphate buffer, pH 6.47. The effluent (flow rate of 50 ml/hr) was collected in 4-ml fractions. (a) After one addition of WSC; (b) after three additions of WSC.

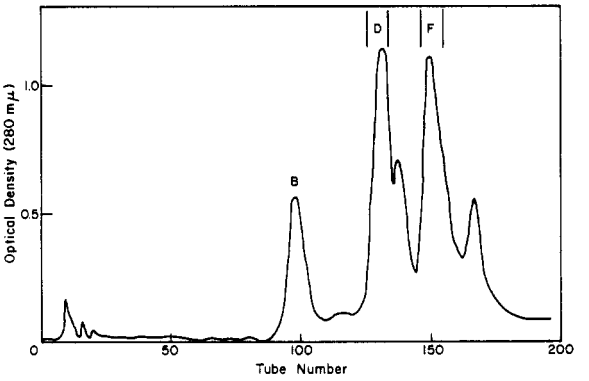


FIGURE 2: Chromatography of the proteins (240 mg) obtained from the reaction between the WSC and ribonuclease. The products were obtained after three additions of the WSC to a ribonuclease solution which contained 5 mg of protein/ml. The methods for the development of the chromatogram were identical with those described for Figure 1.

to 65°. Both cells contained the same solution of a particular ribonuclease component. Thermal equilibrium at each temperature was attained upon allowing the reference solution to stand for 20 to 30 min. The concentration of all protein solutions was determined by assuming a molar extinction coefficient at 278 mμ of 9.78×10^3 (Hermans and Scheraga, 1961).

Potentiometric Titrations. Prior to titration, the last traces of phosphate ion were removed from the proteins by passing a solution of the component under study through a 0.9 × 30 cm column of Amberlite MB-1. The pH of the protein eluent was then lowered to 4.0 by the addition of 0.1 N HCl and the solution was lyophilized. A portion of the lyophilized material was dissolved in 0.15 N KCl, and potentiometric measurements were carried out using a Radiometer Model PHM 4 meter. The meter was equipped with a G222B glass electrode and a Type K100 calomel reference electrode. The meter was standardized against phthalate, borate, tetroxalate, and mixed phosphate buffers which were prepared according to the recommendations of Bates (1954). Titrations were performed with approximately 1 N HCl and carbonate-free 1 N KOH. Carbon dioxide was excluded by continuously purging the titration vessel with water-saturated, carbon dioxide free nitrogen.

GEL FILTRATION. Approximately 10 mg of each protein component was dissolved in 3 ml of 0.2 M phosphate buffer, pH 6.47, and placed on a 1.8 × 140 cm column of Sephadex G-75 which had been equilibrated with the same buffer. The chromatogram was developed with the phosphate buffer at a flow rate of 8 ml/hr.

HYDROLYSES STUDIES. Small amounts of each ribonuclease component (5–10 mg) were dialyzed in the cold overnight against 0.01 M sodium borate buffer, pH 9.5. The protein solutions were then dialyzed against water and lyophilized. Portions of the lyophilized material were chromatographed on analytical columns.

In other experiments, approximately 50 mg of components F, G, and H was dissolved in 5 ml of 0.15 N KCl and the solutions were placed in the Radiometer Model PHM 4 meter. Again, carbon dioxide was excluded by continuously purging the titration vessel with water-saturated carbon dioxide free nitrogen. After flushing the titration cell (equilibrated at 25°) for 4 hr, the protein solutions were rapidly raised to pH values approximating 10 by the addition of 1 N KOH. The solutions were then maintained at the known pH until the rate of base uptake had become very slow.

PERFORMIC ACID OXIDATION. Oxidation of the components and of ribonuclease A was performed at −10° according to the procedure described by Hirs (1956).

TRYPTIC AND PEPTIC DIGESTIONS. The oxidized materials were digested by trypsin for 20 hr according to the procedure described by Hirs *et al.* (1956). Peptic digestions of the oxidized proteins were carried out for 24 hr according to the procedure outlined by Bailey *et al.* (1956).

SEPARATION OF PEPTIDES ON A DOWEX 50-X2 COLUMN. The procedures employed for the chromatography of the tryptic and peptic digests were similar to those employed by Hirs *et al.* (1956) and Bailey *et al.* (1956) with the modifications of Cha and Scheraga (1963) and Riehm *et al.* (1965).

AMINO ACID ANALYSES. Analyses were performed with the Technicon amino acid analyzer. All hydrolyses were carried out in 6 N HCl in sealed, evacuated am-

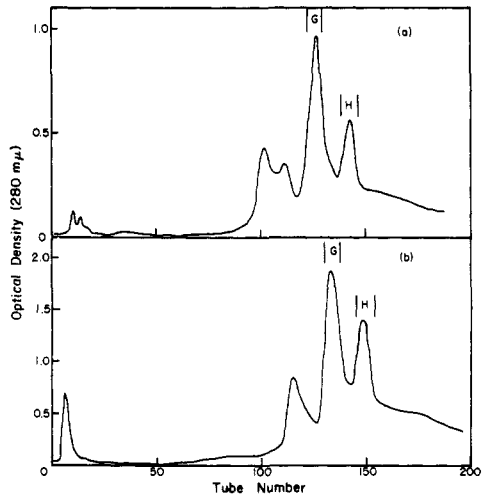


FIGURE 3: Chromatography of the proteins obtained from the reaction between the WSC and ribonuclease. A four-stage gradient was employed; the first three stages contained 200 ml of 0.15 M sodium phosphate buffer, pH 7.0, while the fourth stage contained 200 ml of 1 M sodium phosphate buffer, pH 7.0. (a) After six additions of WSC; (b) after nine additions of WSC.

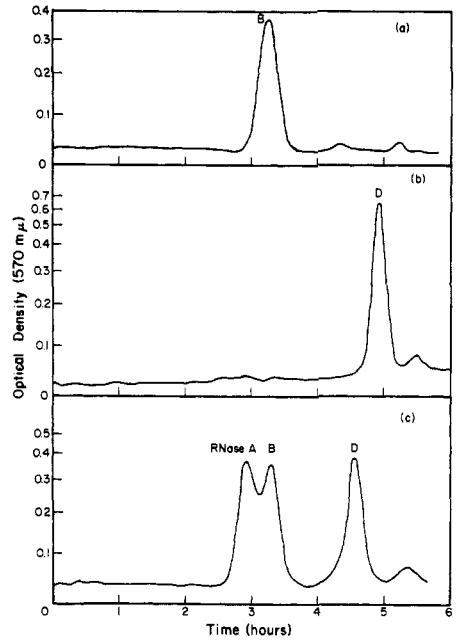


FIGURE 4: Analytical rechromatography of (a) component B, (b) component D, and (c) components B, D, and ribonuclease A. A four-stage gradient was employed; the first three stages contained 35 ml of 0.15 M sodium phosphate buffer, pH 6.40, while the fourth stage contained 35 ml of 1 M sodium phosphate buffer, pH 6.47. The ninhydrin color value of the effluent (flow rate of 20 ml/hr) was obtained with the aid of a Technicon Autoanalyzer.

poules for 22 hr at 116°. The amino acid composition of ribonuclease and its derivatives was calculated by assuming the hydrolysate contained the theoretical number (12) of alanine residues. The amino acid composition of each peptide hydrolysate was derived by assuming that an appropriate amino acid was present in the theoretical amount, and the molar value obtained for this amino acid was then used 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. The majority of the peptide analyses was carried out on the accelerated amino acid analyzer.

Results

Preparation and Isolation of Derivatives. When dilute protein solutions (*ca.* 0.5 mg/ml of reaction mixture) were employed to study the reaction between ribonuclease and the WSC, the resulting reaction products were chromatographed as depicted in Figure 1. Figure 1a illustrates the preparative chromatography of the protein mixture (200 mg) obtained after one addition of the WSC. The two major components (designated A and B) were separately pooled, dialyzed against six changes of water which was 0.001 N in HCl, and then lyophilized. The yield of component A (tubes 68–85) was 102 mg while the yield of component B (tubes 106–117) was 34 mg. Figure 1b shows the preparative chromatography of the reaction mixture (200 mg) obtained after three additions of the WSC. Following the dialysis and lyophilization steps, the yield of component A (tubes 84–95) was 21 mg and of component B (tubes 105–117) was 98 mg.

It is noteworthy that component A chromatographed at a position expected for ribonuclease A. The addition of ribonuclease to component A, followed by analytical chromatography, produced a single symmetrical ninhydrin-positive zone. Therefore, component A is believed to be unreacted ribonuclease. It is also interesting to note that the yield of component B depended on the number of additions of the WSC and not on an extended reaction time. Allowing the reaction between ribonuclease A and the WSC (after one addition of the WSC) to proceed for 12 hr instead of 6 did not increase the amount of component B (as determined by analytical chromatography). This fact is probably due to the hydrolysis of the WSC in acid medium to the substituted urea compound.

The reaction between the WSC and the protein at higher ribonuclease A concentrations (5 mg/ml) produced a number of ribonuclease derivatives. Figure 2 shows the preparative chromatography of the reaction products (240 mg) obtained after three additions of the WSC. The two major components, labeled as D and F in the figure, were pooled (those fractions represented by the vertical lines), dialyzed in the manner described

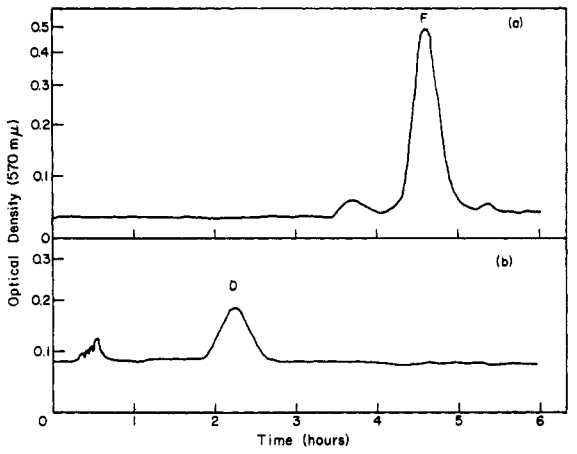


FIGURE 5: Analytical rechromatography of (a) component F, and (b) component D. A four-stage gradient was employed; the first three stages contained 35 ml of 0.15 M sodium phosphate buffer, pH 7.0, while the fourth stage contained 35 ml of 1 M sodium phosphate buffer, pH 7.0. The ninhydrin color value of the effluent (flow rate of 20 ml/hr) was obtained with the aid of a Technicon Autoanalyzer.

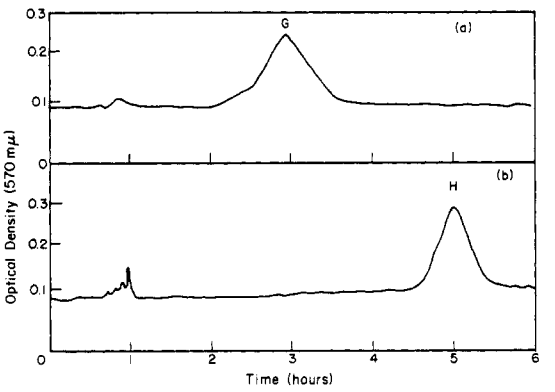


FIGURE 6: Analytical rechromatography of (a) component G, and (b) component H. A four-stage gradient was employed; the first three stages contained 35 ml of 0.3 M sodium phosphate buffer, pH 7.0, while the fourth contained 35 ml of 1 M sodium phosphate buffer, pH 7.0.

previously, and then lyophilized. The yield of component D was 48 mg and of component F was 37 mg. The protein zone eluted at approximately tube 100 was believed to be component B. This protein zone was not isolated.

Figure 3 illustrates the chromatographic patterns obtained from the reaction products after six and nine additions of the WSC. Chromatography of the reaction products (250 mg) after six additions of the WSC is shown in Figure 3a. The two major components, labeled G and H, were pooled (those fractions represented by the vertical lines in the figure), dialyzed, and lyophilized to yield 62 mg of component G and 36 mg of component H. Figure 3b illustrates the chromatographic separation obtained from the reaction mixture (480 mg) after nine additions of the WSC. Subsequent dialysis and lyophilization produced 78 mg of component G and 71 mg of component H.

Purity of Derivatives. The purity of each component was determined by analytical rechromatography. Figure 4 illustrates the analytical rechromatography of component B (Figure 4a) and component D (Figure 4b); these chromatograms indicate that components B and D were quite pure. The small ninhydrin-positive zone appearing near the completion of the chromatograms is believed to be the ammonium ion (Rupley and Scheraga, 1963). The addition of ribonuclease A to a mixture of B and D resulted in the chromatogram shown in Figure 4c. Therefore, neither component appears to contain any detectable quantities of ribonuclease.

Analytical rechromatography of component F is shown in Figure 5a, and it can be seen that this component appears to be comprised of two distinguishable ninhydrin-positive zones. The major component consists of approximately 90% of the total protein. Rechromatography of component D (under conditions identical with those employed for F) resulted in the chromatogram of Figure 5b. The symmetry of the peak again indicates that component D is a single derivative.

The analytical chromatograms of components G and H are shown in Figure 6. Component G (Figure 6a) contained two detectable ninhydrin-positive peaks; the major peak appeared to comprise at least 80% of the total protein. On the other hand, component H (Figure 6b) appeared to be quite symmetrical; this suggests that this derivative is a single species.[4]

Rechromatography of these derivatives indicated that components B, D, and H were quite pure. Components F and G did contain small amounts of impurities. However, since these impurities did not amount to more than 20% of the total protein, repurification procedures were not carried out.

Gel Filtration. The molecular size of each component was determined by chromatography on a Sephadex G-75 column. Figure 7 illustrates the chromatograms obtained for ribonuclease A, component B, and component F. Component B (as with components D, G, and H) appeared as a single ninhydrin-positive zone and in a position which was identical with that observed for ribonuclease. On the other hand component F contained two distinct zones, indicating that this preparation contained a small amount (~12%) of polymerized material. This material may be due to the formation of an intermolecular cross-linked protein or to formation of aggregates which may have occurred during the isolation and subsequent lyophiliza-

[4] Although chromatography is indicative of the purity of a protein, it is possible that many species (either with the same or differing net charge) could chromatograph at the identical position.

TABLE I: Amino Acid Composition of Proteins Obtained from the Reaction between Ribonuclease and the WSC.[a]

Amino Acid	RNAase (theory)	RNAase (obsd)	B	D	F	G	H	Derivative Obtained at High pH
Aspartic acid	15	14.7	15.4	15.4	15.3	15.4	14.5	15.4
Threonine	10	9.5	10.3	10.0	9.9	10.2	10.3	9.9
Serine	15	14.4	15.3	15.1	15.0	15.3	15.5	14.8
Glutamic acid	12	11.3	12.1	11.8	11.4	11.9	11.9	11.7
Proline	4	3.7	3.9	4.3	3.8	3.5	3.6	3.8
Glycine	3	2.7	2.9	2.7	2.8	2.7	2.7	2.7
Alanine[b]	12	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Half-cystine	8	8.0	7.8	7.7	7.4	7.6	8.2	7.7
Valine	9	8.9	8.9	8.7	8.9	8.6	8.8	8.8
Methionine	4	3.4	3.8	3.9	3.6	3.9	3.6	4.0
Isoleucine[c]	3	2.4	2.4	2.1	2.4	2.4	2.3	2.4
Leucine	2	1.8	1.9	1.7	1.9	1.8	1.9	1.8
Tyrosine	6	5.7	5.7	5.6	5.5	5.6	5.9	5.1
Phenylalanine	3	2.6	2.9	2.8	2.7	2.7	2.8	2.6
Lysine	10	9.9	9.8	9.9	10.2	10.4	10.2	4.3
Histidine	4	3.4	3.6	3.7	3.7	3.7	3.7	3.3
Arginine	4	4.1	4.4	4.4	4.3	4.3	4.3	4.2

[a] Moles of amino acid/mole of protein. [b] Assumed as reference. [c] Isoleucine is known not to be completely liberated in a 22-hr hydrolysate.

tion steps. Figure 7d shows the chromatography of a ribonuclease A preparation which was lyophilized from 50% acetic acid. Again, this chromatogram contains two distinct ninhydrin-positive zones. It is known that lyophilization of ribonuclease from a 50% acetic acid solution results in aggregation (Crestfield *et al.*, 1962), and this chromatogram is included to illustrate the separation of monomer from aggregates achieved in the present investigations.

Amino Acid Analyses. Table I reports the amino acid analyses of all components and of ribonuclease A and the protein obtained upon treating ribonuclease with the WSC at high pH (9.5). These data indicate that all amino acids of components B, D, F, G, and H were present in yields which approximated those obtained from a ribonuclease A hydrolysate. On the other hand, treatment of ribonuclease with the WSC at high pH (9.5) resulted in a product which contained a notable decrease in the lysine content. This protein hydrolysate contained only 4.3 residues of lysine per mole of protein compared to 9.8–10.4 lysine residues observed for the other hydrolysates. The yields of tyrosine and histidine were also slightly lower than the yields obtained from the other hydrolysates. However, it is not known if these latter differences are significant.

Enzymatic Activities of the Derivatives. The enzymatic activity of each component is reported in Table II. All components possessed activity toward the RNA substrate; however, the per cent activity (for the various components) differed over a large range. It can be seen that component B was fully active, whereas component H was nearly inactive. The remaining derivatives possessed activities ranging from 60 to 15%. It is concluded that the group(s) of ribonuclease which has reacted to form component B is not required for enzymatic activity. On the other hand, the decreased activities of the remaining derivatives suggest that these components possess conformations which are significantly different from the native molecule, or perhaps reaction has occurred at a group(s) which is required for enzymatic activity. Another possibility is that these derivatives possess a pH for optimum activity which is significantly changed from that of ribonuclease.

TABLE II: Enzymatic Activity of the Components Obtained from the Reaction between Ribonuclease and the WSC.

Derivative	Activity (%)
Ribonuclease A	100
B	97
D	58
F	21
G	16
H	2

Spectrophotometric Titrations. Figure 8 illustrates the spectrophotometric titrations of the tyrosyl residues in ribonuclease A, component F, and component H.

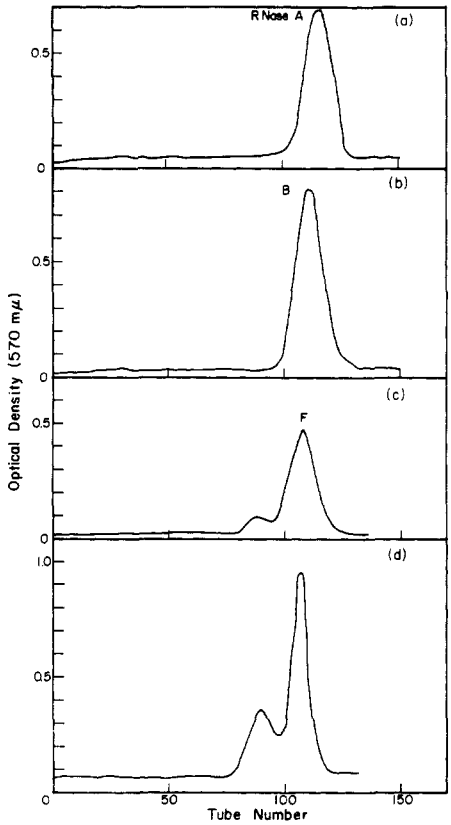


FIGURE 7: Chromatography of protein on a 1.8 × 140 cm Sephadex G-75 column. (a) Ribonuclease A; (b) component B; (c) component F; (d) ribonuclease A which was lyophilized from 50% acetic acid. Fractions (2 ml) were collected at a flow rate of 8 ml/hr and analyzed with the aid of a Technicon Autoanalyzer.

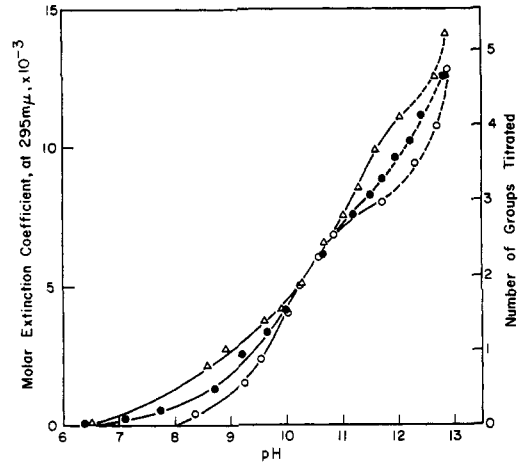


FIGURE 8: Spectrophotometric titration of ribonuclease A (open circles), component F (filled circles), and component H (open triangles) at room temperature. The scale for the number of groups titrated was calculated by assuming an extinction coefficient of 2700 per residue. The dashed lines indicate the time-dependent region of the titration curve. All curves were normalized by assuming the molar extinction coefficient (at 295 mμ) of each derivative to be zero at pH 6.0.

Titrations were also carried out on components B, D, and G. The titration curve for ribonuclease A exhibits the abnormal titration behavior of three of the six tyrosyl groups (Shugar, 1952; Tanford *et al.*, 1955). The titration curves of components B and D were quite similar to that observed for ribonuclease A, thereby suggesting that, under the experimental conditions employed (titration at room temperature), the three abnormal tyrosyl groups in these derivatives have not been perturbed. Component F also appears to contain three abnormal tyrosyl residues; however, the titration of these residues occurs at a slightly lower pH, indicating that this derivative possesses a somewhat "looser" structure than does the native molecule. The tyrosyl residues of component G titrated identically with those of component F. The spectrophotometric titration of component H indicates that this derivative contains only *two* abnormal tyrosines. Therefore, it would appear that the formation of this derivative results in the normalization of one of the abnormal tyrosine residues in ribonuclease. It should be pointed out that the conditions of high pH which are necessary to carry out the spectrophotometric titrations also cause hydrolysis of the derivatives. Thus, due to this hydrolysis, the depicted curves and the reported number of abnormal tyrosines in each derivative may possibly be subject to some error. The hydrolysis, at high pH, is discussed in a later section.

Thermal Transition Studies. Figure 9 shows the thermal transition curves of each derivative and of ribonuclease A. As with ribonuclease, the transitions noted for the derivatives were reversible. It is seen that, although each derivative possesses a lower transition temperature (T_{tr}), at any given pH, than does the native molecule, the shapes of the curves are similar to that reported for ribonuclease A.

Table III reports the optical density changes which occur at pH 6.40 when the derivatives are heated or treated with urea. This table contains a column which indicates the tyrosyl residues of the derivative which are normalized by such treatment. The values obtained from the urea studies were corrected for the effect of the medium (Bigelow, 1960). Heat treatment of components B, D, F, and G produced optical density changes of between −1400 and −1500. Within experimental error, these results agree with the value of −1700 observed for ribonuclease.[5] On the other hand, the observed optical density change noted for component H was notably less negative (500) than that of ribonuclease. The optical density changes of components B, D, F, and G which were observed upon urea treatment were similar to those noted for the native

[5] The lower negative values observed for the derivatives may be due to a slight perturbation of the buried tyrosyl residues.

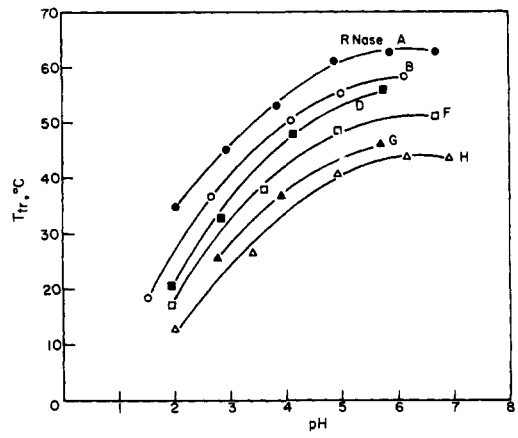


FIGURE 9: Thermal transition temperatures of ribonuclease A and of the five components. The transition temperature was taken as the temperature at which one-half of the total change in the difference extinction coefficient had occurred, between that at 6° and the maximum temperature.

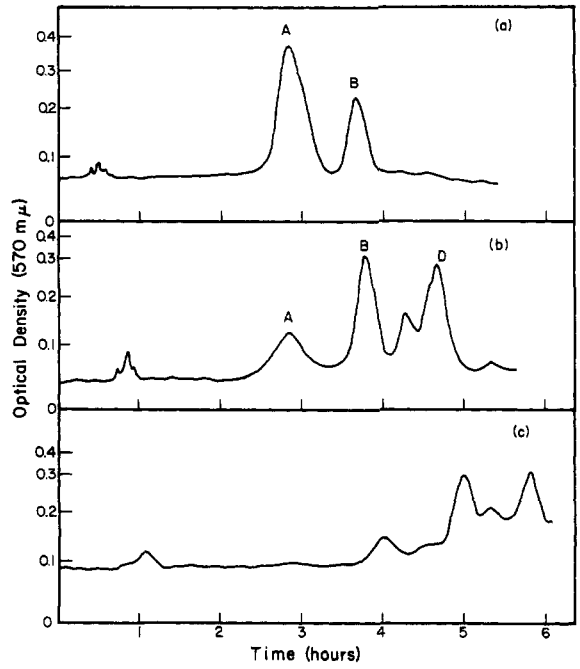


FIGURE 10: Analytical chromatography of protein components which were allowed to stand at pH 9.5 for 12 hr at 2°. (a) Component B; (b) component D; (c) component H. The conditions for developing the chromatograms were identical with those described for Figure 4.

TABLE III: The Change in Optical Density (at 287 mμ) Observed for the Derivatives Formed from the Reaction between Ribonuclease A and the WSC.

Derivative	Treatment	$\Delta\epsilon_{287}$	Residues Normalized
RNAase A	Heat	−1730	A; B
	Urea	−2560	A; B; C
B	Heat	−1480	A; B
	Urea	−2450	A; B; C
D	Heat	−1500	A; B
	Urea	−2470	A; B; C
F	Heat	−1395	A; B
	Urea	−2410	A; B; C
G	Heat	−1410	A; B
	Urea	−2330	A; B; C
H	Heat	−1100	A
	Urea	−2060	A; C

molecule.[5] Urea treatment of component H yielded a molar extinction value which was less negative (500) than that observed for ribonuclease.

Stability of the Derivatives. All components were unstable to alkaline conditions. Figure 10 shows analytical chromatograms of components B, D, and H which were allowed to stand in the cold (2°) at pH 9.5 for 12 hr. Two zones were noted in the chromatogram of component B (Figure 10a). The first zone (denoted as A in Figure 10a) appeared in a position identical with the elution point normally observed for ribonuclease A; the second (denoted as B) appeared at a position which was previously observed for component B. Therefore, alkaline conditions have apparently converted component B to ribonuclease. Figure 10b shows the analytical chromatogram of component D. In this case five zones (note the shoulder on the zone labeled D) were observed. The zones labeled as A, B, and D were believed to be ribonuclease A, component B, and the original component. Figure 10c illustrates the analytical chromatogram of component H. Again, it may be seen that notable degradation of the component has occurred. Chromatography of component H which was allowed to stand in the cold at pH 9.5 for an extended period of time (5 days) resulted in a chromatogram which was similar to that shown in Figure 10c. The relative concentrations of the faster moving components were greater; however, extended alkaline treatment did not result in the conversion of component H to ribonuclease.

Potentiometric Titrations. Table IV reports the number of groups titrated in components B, D, and G from low pH (2.0) to pH 5.0 and 8.0. It would appear from these data that component B differs from ribonuclease A in that one titratable group has been removed below pH 5.0. Component D appears to be a derivative which lacks three titratable groups and G a derivative which lacks four groups. Again, these groups appear to be removed below pH 5.0. It should be pointed out that, in order to obtain meaningful titration values, it was necessary to remove the last traces of phosphate ion from the

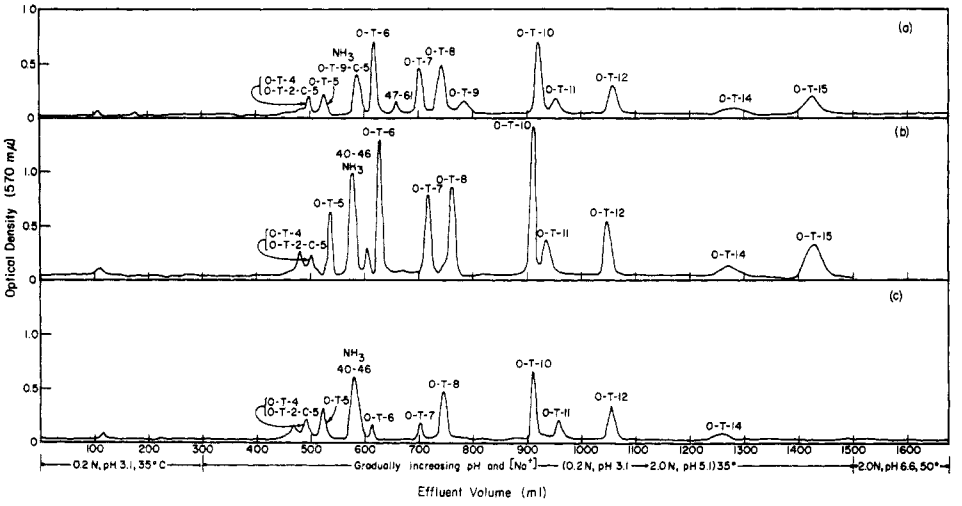


FIGURE 11: Chromatography of peptides from a 20-hr tryptic digest of oxidized protein on a 0.9 × 150 cm column of Dowex 50-X2. The effluent was collected in 2-ml fractions and the ninhydrin color values were obtained with the aid of a Technicon Autoanalyzer. (a) Ribonuclease A; (b) component B; (c) component H.

TABLE IV: The Number of Groups Titrated in Components B, D, and G at 25°.

Component	From pH 2.0 to 5.0	Difference	From pH 2.0 to 8.0	Difference
RNAase A	10.8		16.1	
B	9.6	1.2	14.7	1.4
D	8.2	2.6	13.4	2.7
G	7.1	3.7	11.4	4.1

protein preparations by passing the protein solutions through an MB-1 column. Analytical chromatography indicated that component B was not affected by such treatment. On the other hand, notable changes in the elution patterns of D and G were observed. Thus, MB-1 treatment of component D, followed by analytical chromatography, showed that approximately 70% of the protein was eluted as D. The remainder chromatographed in positions intermediate to the elution positions of ribonuclease A and component D. Analytical chromatography of G which was subjected to MB-1 treatment showed that only 50% of the protein was eluted as G. It is concluded that MB-1 treatment of components D and G causes appreciable hydrolysis. This hydrolysis may be due to the elution of the proteins from the column at the isoionic points. Since the isoionic points of these derivatives would be high, as with ribonuclease, subsequent hydrolysis could occur. Another possibility is that the deionization process produces regions within the resin of high pH. Since hydrolysis was a notable occurrence in the case of components D and G, it was not deemed advisable to carry out titrations on components F and H.

Hydrolyses Studies. Since alkaline conditions cause hydrolysis of the components, studies were initiated to determine the base uptake when components F, G, and H were allowed to stand at pH values approximating 10. Table V shows the equivalents of base which were

TABLE V: The Observed Base Uptake upon Allowing Components F, G, and H to Stand at High pH at 25°.

Component	Moles of Base Uptake/ Mole of Protein
F	3.7
G	4.6
H	6.3

necessary to maintain a constant pH. Periodic and accurately measured amounts of NaOH were added until the base uptake had become very slow. Although the time to obtain this condition was quite extended (36 hr), treatment of a ribonuclease A solution under similar conditions resulted in a base uptake of only 0.25 equiv per mole of protein. These data indicate that basic treatment of components F, G, and H results in a base uptake of 4, 5, and 6 moles, respectively, per mole of protein.

Tryptic Digestions. Figure 11 shows the peptide elution patterns obtained from tryptic digestions of oxidized ribonuclease A and of oxidized components B and H. The elution patterns of oxidized ribonuclease

TABLE VI: Yields of Peptides (in per cent) Obtained from Tryptic Digestion of Oxidized Proteins.

Peptide	RNAase	B	D	F	G	H	Side-Chain Carboxyl Group
O-T-2–C-5	44	22	34	40	45	33	Asp-83
O-T-4	44	20	29	37	41	39	Asp-14
O-T-5	68	91	71	62	58	66	None
40–46	24	17	24	24	15	31	None
O-T-6	83	63	84	46	17	12	Glu-86
O-T-7	63	74	67	73	68	16	Asp-38
47–61	23	Absent	Absent	Absent	Absent	Absent	Glu-49; Asp-53
O-T-8	98	92	87	94	88	86	None
O-T-9	23	Absent	Absent	Absent	Absent	Absent	Glu-49; Asp-53
O-T-10	89	71	74	93	86	86	Glu-2
O-T-11	65	51	64	70	77	76	None
O-T-12	55	68	62	73	75	78	None
O-T-14	31	37	29	32	44	30	None
O-T-15	48	43	35	18	Absent	Absent	Glu-9

A (Figure 11a) was similar to previous reports except that (1) O-T-2[6] was not found, and (2) peptide zones were noted which indicated the presence of traces of chymotrypsin in the trypsin preparation. Thus, the peptide zone ascribed to O-T-4 contained large amounts of O-T-2–C-5 (residues 80–85) and the ammonium ion zone contained O-T-9–C-5 (residues 40–46). In addition, a peptide zone between O-T-6 and O-T-7 analyzed as residues 47–61. It is also noteworthy that O-T-16 was not observed in the chromatogram, an observation which has been previously reported from this laboratory (Cha and Scheraga, 1963).

Figure 11b shows the elution pattern of a tryptic digest of oxidized component B. This chromatogram differed from that obtained for the tryptic digest of oxidized ribonuclease in that O-T-9 (residues 40–61) and the zone appearing midway between O-T-6 and O-T-7, which appears to be residues 47–61, were absent. The peak just prior to O-T-6 analyzed as residues 2–7 (Hirs, 1962).

The elution patterns obtained from tryptic digestions of oxidized components D, F, and G are not shown. However, the pattern obtained for oxidized component D was similar, in every respect, to that shown for oxidized component B. The elution pattern obtained for oxidized component F indicated that the ninhydrin color yields of O-T-6 (residues 86–91) and O-T-15 (residues 8–10) were low relative to the other peptides in this pattern. In addition, O-T-9 and the zone ascribed to residues 47–61 were absent. The elution pattern obtained from the tryptic digestion of oxidized component G showed the absence of O-T-9, the zone ascribed to residues 47–61, and of O-T-15. The ninhydrin color yield of O-T-6 relative to the remaining peptides was quite low.

Figure 11c illustrates the elution pattern of a tryptic digest of oxidized component H. It may be seen that O-T-9, O-T-15, and the zone ascribed to residues 47–61 are absent. In addition, the ninhydrin color yields of O-T-6 and O-T-7 (residues 38–39) are quite low.

Table VI shows the yields obtained from amino acid analyses of the tryptic peptides of each component. Since O-T-2 (residues 67–85) was not found in any digest, the yields of O-T-2–C-5 are included in the table. Also included in the table is a column which indicates the side-chain carboxyl groups that are present in the various peptides.

The yields of the peptides obtained from oxidized components B and D (except for the absence of the peptide zone, which is believed to be residues 47–61, and of O-T-9) compared, within experimental error, to the yields of the peptides observed from the ribonuclease digest.

In addition to the absence of O-T-9 in the tryptic digest of oxidized component F, this digest contained low yields (compared to a ribonuclease digest) of O-T-6 and O-T-15. The yields were approximately one-half of the normally expected amounts. All other peptides in the tryptic digest of oxidized component F were present, within experimental error, in amounts which approximated those found for the tryptic digest of oxidized ribonuclease.

The yields of peptides obtained from a tryptic digest of oxidized component G indicated that, in addition to the complete absence of residues 47–61 and of O-T-9 and O-T-15, peptide O-T-6 was obtained in an amount which was one-fifth of that obtained from the oxidized ribonuclease digest. The yields of the remaining pep-

[6] The terminology employed for the identification of the peptides is that of Hirs *et al.* (1956) for the tryptic peptides, Bailey *et al.* (1956) for the peptic peptides, and Hirs (1960) for the chymotryptic peptides.

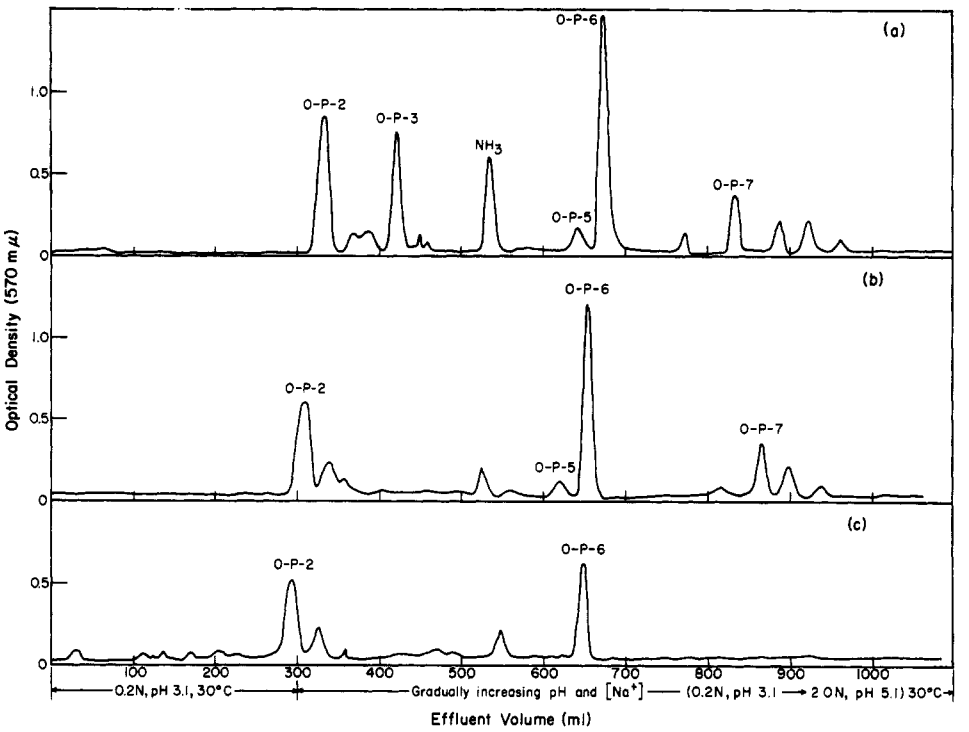


FIGURE 12: Chromatography of peptides from a 24-hr peptic digest of oxidized protein. The chromatographic conditions are the same as in Figure 11 except for the temperature. (a) Ribonuclease A; (b) component B; (c) component H.

tides compared favorably to the yields observed for the ribonuclease digest.

The yields of peptides obtained from a tryptic digest of oxidized component H were similar to those obtained for component G, except that O-T-7 was present in only 16% yield. As may be seen from Table VI, O-T-7 was normally observed in approximately 70% yield.

Peptic Digestions. Figure 12 illustrates the peptide elution patterns obtained from peptic digestions of oxidized ribonuclease A and of oxidized components B and H. The elution pattern of oxidized ribonuclease A (Figure 12a) was similar to that previously reported (Bailey *et al.*, 1956). On the other hand, the elution pattern obtained from the peptic digest of oxidized component B (Figure 12b) differed from that of oxidized ribonuclease in that O-P-3 was not observed and a peptide zone which analyzed as Asp-Val-Glu was eluted just after O-P-2. Figure 12c shows the elution pattern obtained for oxidized component H. In this case O-P-3, O-P-5, and O-P-7 were not found at their normal elution positions. Again, the peptide zone appearing after O-P-2 was observed. Although the elution patterns of the peptic digestions of oxidized components D, F, and G are not shown, these patterns were similar in every respect to that shown for oxidized component H.

Table VII shows the yields of peptides obtained from amino acid analyses of the peptic peptides of each component. Also included in the table is a column which indicates the side-chain carboxyl groups that are present in the various peptides. The yields of peptides obtained from oxidized component B (except for the absence of O-P-3) compared, within experimental error, to the yields of the peptides observed from the ribonuclease digest. The yields of O-P-2 and O-P-6 noted from peptic digestion of oxidized components D, F, G, and H compared, within experimental error, to the yields of these peptides noted from the ribonuclease digest.

Discussion

During this study it has been possible to isolate from the WSC reaction (in relatively large amounts) five ribonuclease derivatives. These components are denoted as B, D, F, G, and H. At least one other derivative was formed (note the component between D and F of Figure 2); however, the yield and separation of this component did not warrant its isolation and characterization. Component B was best prepared under dilute (*ca.* 0.5 mg/ml) protein reaction conditions. Indeed, after three additions of the WSC to the protein solution, ribonuclease was almost entirely converted to this derivative. It is also noteworthy that the reaction between the WSC and ribonuclease in dilute protein solutions produced but a single derivative.

To obtain other derivatives, the concentration of the protein in the reaction mixture was increased tenfold. Also, by employing these higher protein concentrations, reaction mixtures were isolated and chromatographed after three, six, and nine additions of the WSC. It is

TABLE VII: Yield of Peptides (in per cent) Obtained from Peptic Digestion of Oxidized Proteins.

Peptide	RNAase	B	D	F	G	H	Side-Chain Carboxyl Group
O-P-2	95	78	85	87	75	76	Asp-121; Val-124
Asp-Val-Glu	Absent	33	48	45	NA[a]	26	Asp-53
O-P-3	65	Absent	Absent	Absent	Absent	Absent	Asp-53
O-P-5	16	17	Absent	Absent	Absent	Absent	Glu-111
O-P-6	84	79	72	58	60	72	Glu-2
O-P-7	26	18	Absent	Absent	Absent	Absent	Glu-49

[a] NA = not analyzed.

interesting to note that the chromatographic pattern of the reaction products obtained after nine additions of the WSC resembled that of the products obtained after six additions. Only the relative amounts of components G and H varied. Therefore, component H appears to be the end product of this reaction.[7] It should also be pointed out that isolations of the various components were carried out a number of times. Chromatography of the products obtained after nine additions of the WSC has been performed on five separate occasions. In every instance, the chromatograms were similar to those depicted above.

Possible Reactions between a Protein and a Water-Soluble Carbodiimide. The reactions between a carbodiimide and the functional groups of a protein could be both numerous and complex. The reaction between the reagent and a nucleophilic group, such as an amino or imidazole group, in the protein should yield amino acid derivatives which would be expected to be stable to acid hydrolysis. In the case of amino groups, a guanidine derivative would be formed. Amino acid analyses of the products obtained from the reaction at pH 4.5 (components B, D, F, G, and H) indicated that all components possessed amino acid compositions which were similar to those found for a ribonuclease hydrolysate. Therefore, it would appear that the reaction between ribonuclease and the carbodiimide, at low pH, does not involve direct reaction between the reagent and amino or imidazole groups in the protein.[8] This observation might be expected since at pH 4.5 most (if not all) of these groups would be protonated. On the other hand, the reaction between the protein and the water-soluble carbodiimide at high pH (9.5), followed by amino acid analyses, resulted in a product which contained fewer lysine residues than did a ribonuclease hydrolysate. Therefore, it is concluded that the reaction between a protein and a water-soluble carbodiimide, at high pH, results in the guanidination of ϵ-amino groups. It should be pointed out that the guanidine derivative was not found in the chromatograms.

Carbodiimides have also been reported to react with alcohols to form pseudourea ethers. Thus, the side-chain hydroxyls of threonine, serine, and tyrosine residues could also be susceptible to attack. However, reactions of this type require conditions which are quite vigorous (Dains, 1899; Khorana, 1954). In addition, Dains reported that the action of aqueous HCl upon these ethers produces decomposition to alkyl chlorides and the substituted urea compounds. Thus, any pseudourea ether formation should lead to a decrease in the threonine, serine, or tyrosine content of the protein upon acid hydrolysis and amino acid analysis. As was noted previously, the content of all amino acids in hydrolysates of components B, D, F, G, and H compared favorably to the yields obtained for a ribonuclease hydrolysate.

The data presented in the Results section strongly support the hypothesis that the isolated derivatives arise from carboxyl group reaction. All conceivable carboxyl group reactions (those discussed below) would lead to acid-labile products. As mentioned previously, amino acid analyses indicate that these components are labile to 6 N HCl. The titration data of components B, D, and G indicated that carboxyl groups had been removed. Although the hydrolysis problem introduces errors into an accurate evaluation of the number of titratable groups removed, there appears to be little doubt that groups, which in the native molecule titrate below pH 5.0, are removed in components B, D, and G. Also, since carboxyl groups would be expected to titrate in the region below pH 5.0, it is believed that these components arise from carboxyl group reaction. In addition, since the hydrolysis problem was not a major one in the determination of the number of titratable groups in components B and D, it is believed that B is a derivative which is produced upon the reaction of one carboxyl group and D a derivative which is produced upon reaction of three carboxyl groups. The large amount of hydrolysis noted for G (50%) does

[7] As is seen in Figure 3, protein is eluted after component H. However, no distinct zones were noted.

[8] If reaction between amino and imidazole groups has occurred, amino acid analyses indicate that this type of reaction must be negligible.

not make it possible to state the exact number of carboxyl groups which have been removed. However, since the difference in the number of titratable groups (between G and ribonuclease A) approximates four groups, it is believed that component G is a derivative which contains at least four reacted carboxyl groups which are free in the native molecule.

The general instability of all components to mild alkaline treatment may also be explained by carboxyl group reaction. Since hydrolysis at alkaline pH of modified carboxyl groups would be expected to require a base uptake to maintain a constant pH, it is believed that components F, G, and H are ribonuclease derivatives which have been modified at 4, 5, and 6 carboxyl groups, respectively. These components were the derivatives which were subjected to the hydrolysis experiments. However, it should be pointed out that, due to the length of time required to carry out the hydrolysis experiments, these numbers could be in error. The peptide chromatography data are also consistent with carboxyl group reaction. Certain peptides, which in an oxidized ribonuclease digest are known to contain side-chain carboxyl groups, were either absent or present in very small amounts. On the other hand, those peptides which do not contain side-chain carboxyl groups were found in amounts comparable to the yields obtained from an oxidized ribonuclease digest.

According to the generally accepted mechanism of interaction between an organic acid and a carbodiimide (Khorana, 1955), the carbodiimide is initially protonated. This is then followed by attack of a carboxylate anion to form an unstable acylisourea. The activated carboxyl group (the acylisourea) may then undergo an acyl shift to form a stable acylurea, or come under attack from a nucleophile (such as an amine, alcohol, or carboxylate anion) to form an amide, ester, or anhydride. In addition to the above mentioned possibilities, Franzblau *et al.* (1963) suggest that the acylisourea could cyclize. In the case of an aspartic acid residue in an α-peptide linkage, cyclization would yield a succinimide ring, and a glutamyl residue in an α-peptide linkage could form either a glutarimide or acylpyrrolidone ring.

The evidence presented in the Results section suggests that amide formation has not occurred, or at the most it has occurred to a negligible degree. Tryptic digestions of every oxidized component indicated that proteolytic cleavages had occurred at all susceptible lysine residues. Also, the fact that each derivative is unstable to alkaline conditions indicates that amide formation has not taken place. Although the formation of acid anhydrides and esters cannot be definitely excluded, it is believed that such reactions have not occurred during the formation of the five ribonuclease derivatives. Acid anhydrides are excluded because of their general instability in aqueous medium. The chromatography and dialysis steps in the isolations involved solutions of these derivatives for extended times. Ester formation is excluded since ribonuclease is believed not to possess any reactive hydroxyl groups (as do various esterases) and by the fact that it was not necessary to block the hydroxyl groups in the formation of various threonine- and serine-containing peptides (Sheehan and Hess, 1955; Sheehan *et al.*, 1956). It is also noteworthy that Sephadex chromatography indicated that all derivatives were, in the main, monomeric in size. Therefore, even if these derivatives did contain small amounts of ester, amide, or anhydride linkages, these bonds must be of an intramolecular nature.

The most probable reaction products to be obtained from the reaction between a protein and a water-soluble carbodiimide (at low pH) are either imides or acylureas. All experimental evidence presented in this communication is in agreement with this assumption. Thus, the monomeric size of the components, the general instability to mild alkaline conditions, the removal of groups which titrate (in native ribonuclease) below pH 5.0, and the peptide chromatography data suggest that either acylurea or imide formation (or both) give rise to the observed derivatives. Certain experimental evidence favors the acylurea hypothesis while other data indicate that imide formation may have occurred. It was noted previously that peptide analyses of the various components showed the complete (or nearly complete) absence of certain peptides which in a ribonuclease digest contain side-chain carboxyl groups. However, in no case were the altered peptides found. This fact can best be explained by assuming acylurea formation since, in addition to removing a negative charge from a certain peptide, the urea moiety contains a quaternary amine grouping. Therefore, due to the increase in positive charge, these peptides would most probably be strongly adsorbed to the Dowex 50-X2 column. Also, the nonpolar portion of the urea moiety (the cyclohexyl grouping) could contribute to the strength of adsorption to the resin. On the other hand, it is also possible although not as probable that imide formation could yield peptides which would be strongly adsorbed to the resin. It is noteworthy that peptic digests of all oxidized components showed the presence of a peptide zone which analyzed as Asp-Val-Glu. This peptide zone appeared in the elution pattern just after O-P-2 and was not observed in the elution patterns of oxidized ribonuclease digests. The only sequence in ribonuclease which would be compatible with the analysis of this peptide is residues 53–55. In this case, due to the elution position of the peptide, it would seem plausible to assume that reaction at Asp-53 results in succinimide formation with the nitrogen of Val-54 and that subsequent peptic digestion occurs at the Ala_{52}-Asp_{53} peptide bond instead of at the Leu_{51}-Ala_{52} bond. The latter bond is the bond normally hydrolyzed by the catalytic action of pepsin.

The fact that component H was not converted to ribonuclease upon mild alkaline treatment for an extended reaction time would support the imide hypothesis since hydrolysis of an imide bond could occur in one of two ways. That is, hydrolysis of an imide could result in the formation of the free α-carboxyl group instead of the free γ-glutamyl carboxyl or the β-aspartyl carboxyl group. Of course, it may also be possible that

acylureas hydrolyze by means of an imide intermediate, or in a manner similar to that proposed by Khorana (1952).

It should be emphasized that, even though the exact nature of the reaction products is not known, the important points are (1) there appears to be little doubt that each component arises from carboxyl group reaction, and (2) reaction at these carboxyl groups does not result in a polymerized product. In addition, the arguments presented above indicate that the derivatives are due to either imide and/or acyl urea formation.

Abnormal Tyrosines. Spectrophotometric titrations of components B, D, F, and G indicated that these proteins contained three abnormal tyrosines. On the other hand, component H appears to contain only *two* abnormal tyrosines.

It has been postulated by Hermans and Scheraga (1961) that the thermal transitions of ribonuclease are due to a gross unfolding of the molecule such that one or more of the abnormal tyrosyl residues are brought from a nonpolar environment into a polar one. In addition, since these transitions occur over the acid range, these workers have proposed that the abnormal tyrosyl residues are near carboxylate ions. Since each derivative undergoes reversible transitions, it is believed that each component possesses one or more abnormal tyrosyl residues which are near carboxylate ions. The lower T_{tr} which was observed for all derivatives would suggest that the abnormal tyrosyl residues are not as deeply buried in their nonpolar environment as they are in the native molecule, and, in derivative H, one of the tyrosyl residues has become completely exposed to the solvent.

From the changes in the optical density which occur at 287 mμ during these transitions, Bigelow (1960, 1961) has assigned molar extinction values to the contributions of each of the three abnormal tyrosyl residues in ribonuclease. Bigelow has defined these residues as A, B, and C with molar extinction values of -1000, -700, and -1000, respectively. Heating a ribonuclease solution (at neutral pH or lower) results in a $\Delta\epsilon_{287}$ of -1700 and the normalization of tyrosyl residues A and B. Alkaline or urea treatment produces a $\Delta\epsilon_{287}$ of -2600 and the normalization of all three residues.

Treatment of components B, D, F, and G with urea or heating these proteins at neutral pH (6.40) resulted in molar extinction values which were similar to those obtained from identical studies on ribonuclease. Therefore, it is concluded that these proteins contain three abnormal tyrosine residues. On the other hand, heating component H at neutral pH or treating this protein with urea produced molar extinction values which were approximately 500 less negative than the values observed for the ribonuclease molecule. These observations suggest, according to the proposals of Bigelow (1960, 1961), that the abnormal tyrosyl residue B of ribonuclease is normalized in component H.

Identification of the Carboxyl Groups Which Have Undergone Reaction. The identification of the carboxyl groups which underwent reaction (in any given derivative) was determined from tryptic and peptic digestions of the oxidized proteins. Tryptic digestion can yield information on eight of the eleven carboxyl groups known to be present in ribonuclease. O-T-2[9] contains Asp-83; O-T-4, Asp-14; O-T-6; Glu-86; O-T-7, Asp-38; O-T-9, Glu-49 and Asp-53; O-T-10, Glu-2; and O-T-15, Glu-9. Peptic studies can yield information on the remaining three carboxyl groups. O-P-2 contains Asp-121 and Val-124 and O-P-5 contains Glu-111. In addition, since O-P-3 contains Asp-53 and O-P-7 Glu-49, it is possible to determine unambiguously the state of the two carboxyl groups which are to be found in O-T-9. Thus, the absence or low yield of a peptide which is known to contain a side-chain carboxyl group is believed to be evidence that the carboxyl group in that peptide has undergone reaction.

Except for the absence of peptide O-T-9 and the peptide zone ascribed to residues 47–61, the yields of peptides and the elution pattern of a tryptic digest of oxidized component B appeared similar to the yields of peptides and the elution pattern obtained from the oxidized ribonuclease digest. Therefore, it is believed that either Asp-53 or Glu-49 (or both) have been modified. A peptic digest of oxidized component B indicated that O-P-3 (containing Asp-53) was not eluted at its normal position. All other peptic peptides were noted at their normal elution position. Therefore, it is concluded that component B differs from ribonuclease in that only Asp-53 has been modified.

The yields of peptides and the elution patterns obtained from a tryptic digest of oxidized component D were similar in every respect to that obtained from an oxidized component B digest. Again it is concluded that reaction has occurred at either Asp-53 or Glu-49, or at both carboxyl groups. The elution pattern and yields of peptides obtained from a peptic digest of this component indicated that reaction had occurred at Asp-53, Glu-49, and Glu-111. The peptides O-P-3 (containing Asp-53), O-P-7 (containing Glu-49), and O-P-5 (containing Glu-111) were not observed. The remaining two well-defined peptic peptides (O-P-2, containing Asp-121 and Val-124; and O-P-6, containing Glu-2) were found in positions identical with those for the oxidized ribonuclease digest.

The yields of peptides and the elution positions of peptides obtained from tryptic and peptic digests of oxidized component F were similar to those obtained from oxidized component D with the exception that O-T-6 (containing Glu-86) and O-T-15 (containing Glu-9) were found in yields which were approximately one-half of the amounts normally observed. It is reasonable to assume that component F is a mixture of two derivatives, half of this component being a derivative which has reacted at Asp-53, Glu-49, Glu-111, and Glu-86, and the remaining half a derivative which has reacted at Asp-53, Glu-49, Glu-111, and Glu-9.

[9] Since the small amount of chymotrypsin impurity in the trypsin preparation caused total digestion of O-T-2, even in the oxidized ribonuclease digest, the presence or absence of O-T-2-C-5 (residues 80–85) has been used as the criterion for determining the state of Asp-83.

The elution patterns and yields of peptides obtained from the tryptic and peptic digestions of oxidized component G indicate that this derivative is formed upon reaction at Asp-53, Glu-49, Glu-111, and Glu-9. This reasoning is due to the observations that O-T-9, O-T-15, and the peptide zone ascribed to residues 47–61 were absent from the tryptic digest and O-P-3, O-P-5, and O-P-7 were absent from the elution pattern of the peptic digest. The small amount of O-T-6 (17% as compared to approximately an 80% yield in the ribonuclease digest) indicates that component G has reacted to a large extent at Glu-86. The small amount of O-T-6 noted in this elution pattern demonstrates that the component was not a single derivative. As reported previously, component G did contain a small amount of impurity, as determined from analytical chromatography.

Peptide studies which were carried out on oxidized component H indicate that, as with component G, reaction has occurred at Asp-53, Glu-49, Glu-111, and Glu-9. In addition, the low yields of O-T-6 and O-T-7 (relative to the expected yields) indicated that this component has also undergone reaction, in the main, at Glu-86 and Asp-38. Again, the small amounts of O-T-6 and O-T-7 observed in the elution patterns of tryptic digests of this component suggest that, even though component H chromatographed as a single entity, it does contain small amounts of impurities. Therefore, it is recognized that component H probably contains small amounts of derivatives which have reacted at carboxyl groups other than at Asp-38 and Glu-86.

The peptide studies indicate that component B is a derivative which has been modified at Asp-53, and component D a derivative which possesses three modified carboxyl groups (Asp-53, Glu-49, and Glu-111). Component F appears to be a mixture of two derivatives, half of which possesses Asp-53, Glu-49, Glu-111, and Glu-86 modified and the remainder a derivative which has been modified at Asp-53, Glu-49, Glu-111, and Glu-9. Although components G and H contain small amounts of impurities, it is believed that component G has been modified (to a large degree) at Asp-53, Glu-49, Glu-111, Glu-86, and Glu-9, while component H has been modified at Asp-53, Glu-49, Glu-111, Glu-86, Glu-9, and Asp-38.

Structural Implications. The thermal transition studies, in the acid range, indicate that reaction at carboxyl groups results in a perturbation of the abnormal tyrosyl residues. Thus, reaction at only one carboxyl group (Asp-53 in component B) causes a decrease of approximately 5° in the observed T_{tr}. As more carboxyl groups react, the observed T_{tr} values become lower. Components G and H which contain five and six reacted carboxyl groups, respectively, possess transition temperatures which are 20–25° lower than those observed for ribonuclease. Therefore it is concluded that most, if not all, of the carboxyl groups perform a vital role in maintaining the conformation of the protein in solution. These results are in contrast to those observed upon chemical modification of amino groups in this protein (Riehm and Scheraga, 1966). In the latter case, cyanoethylation of all ϵ-amino groups caused only a slight decrease in T_{tr}. It is also interesting to note that the enzymatic activity of all components (except component B) which were obtained from the reaction between the carbodiimide and ribonuclease were significantly lower than that observed for the native molecule. This observation could, in part, be due to gross conformational changes occurring near the active center of the protein.

Component B appears to be the initial product of the reaction, and since it is believed to be a derivative which has reacted only at Asp-53 it is concluded that, of all the carboxyl groups in ribonuclease, Asp-53 is the most reactive toward chemical modification. This result is in agreement with a previously published report (Riehm and Scheraga, 1965). Since significant conformational changes do occur upon the reaction at Asp-53, it is not possible to state which of the remaining carboxyls in the native protein is the next most reactive. That is, reaction at Asp-53 undoubtedly results in conformational changes within the molecule (particularly around Asp-53), and these changes in conformation could dictate which carboxyl group would react next.

Spectrophotometric titration data and changes in the optical density at 287 mμ which occur during the thermal transition indicate that components B, D, F, and G contain three abnormal tyrosyl residues. Therefore, although reaction at certain carboxyl groups (those groups which give rise to these components) causes notable conformational changes, reaction at Asp-53, Glu-49, Glu-111, Glu-9, and Glu-86 (the reacted carboxyl groups of component G) does not normalize any of the abnormal tyrosyl residues. On the other hand, from spectrophotometric titrations and thermal transition studies, component H appears to contain only two abnormal tyrosine residues. The normalized tyrosine is tyrosyl residue B, according to the proposals of Bigelow (1961). Peptide chromatography of tryptic and peptic digests indicates that component H differed from component G only in the very low yield of O-T-7 observed from a tryptic digest of oxidized component H. Therefore, it would appear that reaction at Asp-38 results in the normalization of tyrosyl residue B of ribonuclease. It is interesting to note that Asp-38 was one of the three free carboxyl groups which was found to be present in a partially methylated derivative (Riehm *et al.*, 1965). It is also noteworthy that this partially methylated component appeared, from thermal transition studies (Broomfield *et al.*, 1965), to contain tyrosyl residue B as an abnormal residue. These results strongly support the hypothesis that one or more of the abnormal tyrosyl residues in ribonuclease are involved, in a nonpolar region of the molecule, in specific tyrosyl–carboxylate interactions. Indeed, it would appear that one of the carboxyl groups is Asp-38 and that it is involved in a specific interaction with tyrosyl residue B. However, it should be pointed out that it is not known if reaction at Asp-38 alone would cause the normalization of a tyrosyl residue. It was reported above that, although component G did contain three abnormal

tyrosyl residues, this component had undergone significant conformational alterations. Thus, reaction at Asp-38 may give rise to added conformational changes and these conformational changes could result in the normalization of the tyrosyl residue. In addition, Asp-38 is in a region of high positive charge (Lys-37, Arg-39, Lys-41), and reaction at this carboxyl group could cause a change in the net charge about this region such that repulsion of positive charges could produce large changes in the conformation of the protein.

Component H appears to be the end product of the reaction, at pH 4.5, between the carbodiimide and the protein. Peptide analyses indicated that this protein contained five free carboxyl groups (Glu-2, Asp-14, Asp-83, Asp-121, and Val-124), and it is interesting to speculate as to why these carboxyl groups did not (or at the most to a nondetectable amount) undergo modification. The size of the reagent could impose restrictions on the extent of reaction. Thus, due to steric hindrance, the modification of a particular carboxyl group could be quite slow or perhaps nonexistent. Component H appears to contain Asp-121 and Val-124 as free carboxyl groups. Anfinsen (1961) proposes that the C-terminus of ribonuclease is buried in the protein. If this assumption is correct, then reaction at Val-124 and Asp-121 may be expected to be slow. Electrostatic effects could also limit the extent of reaction. A carboxyl group which is near a region of high positive charge should react slower than one not so placed. This fact may be part of the reason why Asp-38 is the last carboxyl group to react. It may also be a factor as to why Glu-2 and Asp-14 do not appear to react. Glu-2 is adjacent to the N-terminus lysine residue, and Asp-14 is near His-12 and hence is believed to be near His-119 (Crestfield *et al.*, 1963) and Lys-41 (Hirs, 1962). Another possibility is that some of these carboxyl groups are involved, in a nonpolar region of the molecule, in specific tyrosyl–carboxylate interactions (Hermans and Scheraga, 1961). It is not possible from the data presented in this communication to state which of the five carboxyl groups could be near the two abnormal tyrosine residues in component H; however, it is noteworthy that two of the five carboxyl groups (Asp-14 and Asp-83) are two of the three free carboxyl groups which were found to be present in a partially methylated ribonuclease derivative (Riehm *et al.*, 1965). Therefore it is concluded that, if specific tyrosyl–carboxyl interactions do occur in ribonuclease, Asp-14 and Asp-83 (in addition to Asp-38) are the probable carboxyl groups involved.

Further work is in progress to locate the buried tyrosyl groups of component H. This information may enable the pairing of the three buried tyrosyl groups with the appropriate three buried carboxyl groups.

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