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Structural Studies of Ribonuclease. XXII. Location of the Third Buried Tyrosyl Residue in Ribonuclease*

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ABSTRACT: Ribonuclease was iodinated with 6 moles of I_2/mole of ribonuclease at pH 6.7 and 4°. The major component, peak C, was isolated by chromatography on carboxymethylcellulose. Spectrophotometric titrations and amino acid analysis of the iodinated material showed the presence of about three uniodinated and still abnormal tyrosines. The ultraviolet difference spectrum indicated that three tyrosines had been diiodinated.

The oxidized iodinated protein was subjected to enzymatic digestion by trypsin, chymotrypsin, and pepsin, respectively, and the resulting peptides were chromatographed on Dowex 50-X2. Analysis of the peptides showed that Tyr 92, Tyr 97, and Tyr 25 were uniodinated. Structural implications, based on the results of this and the accompanying paper, are presented in the following paper [Li, L.-K., Riehm, J. P., and Scheraga, H. A. (1966), *Biochemistry 5*, 2043].

As an important step in the study of the folding of protein molecules in solution, it is desirable to determine which amino acid side chains are exposed to the solvent and which are buried. In the case of bovine pancreatic ribonuclease (RNAase), an abundance of physical evidence indicates that three of the six tyrosines behave normally while the other three show anomalous titration behavior and are presumably buried (Shugar, 1952; Tanford *et al.*, 1955). The three anomalous tyrosines are not all equivalent as indicated by differences in the spectral shifts occurring under various denaturation conditions (Bigelow, 1961). On the basis of the work of Hermans and Scheraga (1961) and Scott and Scheraga (1963), it appears that the buried tyrosyl residues may be near carboxyl groups and surrounded by nonpolar groups.

Tyrosine residues can be iodinated under relatively mild conditions in aqueous solution, and the iodination of RNAase has been studied by Cha and Scheraga (1963) and by Donovan (1963). They were able to locate, respectively, two and one of the buried tyrosyls in the amino acid sequence. Recently, Riehm *et al.* (1965) have located the three abnormal carboxyl groups of RNAase and have suggested possible pair relationships between these carboxyls and the known buried tyrosyls. Fujioka and Scheraga (1965) have located the single buried tyrosine which remains in pepsin-inactivated RNAase. To complete the picture, it is necessary to locate the remaining buried tyrosine in native RNAase.

The present paper describes the iodination of RNAase under somewhat milder conditions than those used by Cha and Scheraga (1963) and by Donovan (1963). These workers used the conditions recommended by Hughes and Straessle (1952), *i.e.*, pH 9.5 and 0°, to minimize side reactions. We have carried out our iodinations at neutral pH and 4°. At this lower pH, the reaction is much slower, but no evidence of oxidative side reactions was observed.

The product from iodination was fractionated, and the major component was characterized by spectrophotometric titration, ultraviolet difference spectra, and amino acid analysis following performic acid oxidation and acid hydrolysis. Three tyrosyl groups were found to be uniodinated. The positions of these uniodinated groups in the amino acid sequence were de-

* From the Department of Chemistry, Cornell University, Ithaca, New York. *Received February 17, 1966.* 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-4766) from the National Science Foundation.

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termined by analysis of proteolytic digests of the performic acid oxidized material. As found by Cha and Scheraga (1963), performic acid oxidation destroys the iodinated tyrosyl residues, altering the elution properties of those peptides containing iodinated tyrosines.

Proteolytic digests of oxidized RNAase were also fractionated and compared with the digests of our iodinated derivative. All peptides suspected of containing tyrosine were subjected to amino acid analysis, and all new peaks occurring in the elution pattern of the oxidized iodinated derivative were analyzed.

Experimental Section

Materials. Bovine pancreatic RNAase, five times recrystallized, was purchased from Sigma Chemical Co. (Lot No. 114B-1510). It was purified further on unsieved Amberlite IRC-50, XE-64 resin in 0.2 M sodium phosphate buffer, pH 6.47, on a 7.5 × 60 cm column (Hirs *et al.*, 1953). The fraction containing RNAase A was deionized by passage through a 3.3 × 35 cm column of MB-1, mixed-bed resin (Rohm and Haas), lyophilized, and stored at 2° until needed.

Crystalline trypsin (twice recrystallized, Lot 6108), salt-free chymotrypsin (Lot CD1-678-8413), and crystalline pepsin (twice recrystallized, Lot 681) were all purchased from the Worthington Biochemical Corp., and were used without any further purification.

Monoiodotyrosine was purchased from Nutritional Biochemicals Corp., and 3,5-diiodotyrosine from Mann Chemical Co. Formic acid (99% pure) was obtained from Matheson Coleman and Bell while 30% hydrogen peroxide was supplied by Merck and Co.

Sephadex G-25 was supplied by Pharmacia of Sweden. Analytical grade Dowex 50-X2 was purchased from Bio-Rad Laboratories, and it was prepared according to the recommendations of Hirs *et al.* (1956a). Carboxymethylcellulose (CM 11) was obtained from the Whatman Co. The iodinating reagent which was 0.05 M in I_2 and 0.2 M in KI was standardized *vs.* As_2O_3 obtained from Mallinckrodt Chemical Works.

During the experiments it was observed that some tyrosine was being destroyed during the hydrolysis, and the cause was traced to impurities in the HCl. Thus, distilled (constant boiling) HCl, *ca.* 6 N, was used to eliminate this destruction.

Ribonucleic acid was purchased from Nutritional Biochemicals Corp. All other chemicals were reagent grade unless otherwise indicated.

Iodination of Ribonuclease. The iodination was carried out in 1 M ammonium acetate (~pH 6.7). *Ca.* 1 g of RNAase was dissolved in 200 ml of 1 M ammonium acetate. The exact concentration was determined by diluting 1 ml of the solution with 3 ml of 1 M ammonium acetate, and then recording the optical density at 278 mμ. Using an extinction coefficient of 0.715 ml/mg (Hermans and Scheraga, 1961) at 278 mμ the concentration can be evaluated. Exactly 6 moles of iodine/mole of protein was pipetted into the RNAase solution while the mixture was being constantly stirred with a magnetic stirrer. The mixture was allowed to stand for 3 days at 4° during which time the yellow iodine color disappeared. The iodinated RNAase (I_6RNAase) was then transferred into Visking casing, and was exhaustively dialyzed *vs.* distilled water, with numerous changes over a period of 2 days. The I_6-RNAase solution was then lyophilized.

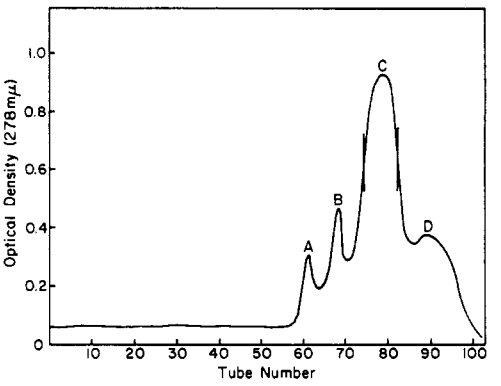

FIGURE 1: Chromatography of iodinated ribonuclease (300 mg) on carboxymethylcellulose. A two-stage linear gradient was employed; the first stage contained 1.2 l. of 0.005 M Tris buffer + 0.001 M EDTA, pH 8.5, while the second stage contained 1.2 l. of 0.005 M Tris buffer + 0.001 M EDTA + 0.15 M sodium acetate, pH 8.5. The flow rate was 60 ml/hr, and the effluent was collected in 10-ml. fractions. The optical density was read at 278 mμ. Fraction C was pooled separately as shown.

Fractionation of Iodinated RNAase. The I_6RNAase was fractionated by chromatography on carboxymethylcellulose (Taborsky, 1959). The columns were equilibrated with 0.005 M Tris, 0.001 M EDTA, pH 8.5, and a linear ionic-strength gradient from this solution to a solution 0.15 M in sodium acetate, 0.005 M in Tris, and 0.001 M in EDTA, pH 8.5, was established. The pH was chosen to provide a maximum charge difference between tyrosine (pK = 9.5), iodotyrosine (pK = 8.5), and diiodotyrosine (pK = 6.5). Analytical scale fractionations were performed on a 0.9 × 30 cm column, and were monitored using a Technicon autoanalyzer by following the ninhydrin color of the eluate. The total volume of eluent in these analytical runs was 600 ml and the flow rate was *ca.* 20 ml/hr. On a preparative scale, 250–350 mg was placed on a 2.5 × 40 cm column and a total volume of 2.4 l. of eluent was used, with a flow rate of *ca.* 60 ml/hr. Fractions of 10 ml each were collected and the optical density was measured at 278 mμ.

Figure 1 shows the results of a typical preparative fractionation. The major fraction, C, was lyophilized to <50 ml. It was then purified by passage through a 2.5 × 50 cm column of Sephadex G-25 equilibrated with 0.05 M ammonium acetate, followed by dialysis of the protein solution *vs.* distilled water and lyophilization.

Spectral Measurements. The molar extinction coefficient of the iodinated derivative at 278 mμ was obtained as follows. First, ninhydrin derivatives of iodinated

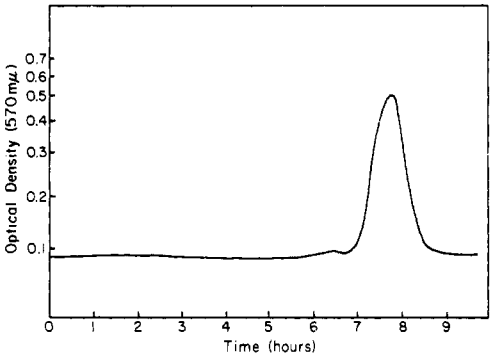

FIGURE 2: Analytical rechromatography of component C. A four-stage gradient was employed. The first three stages consisted of 70 ml of 0.005 M Tris buffer containing 0.001 M EDTA, pH 8.5, while the fourth stage consisted of 70 ml of 0.005 M Tris containing 0.001 M EDTA and 1.0 M sodium acetate, pH 8.5. The ninhydrin color value of the effluent (flow rate of 20 ml/hr) was obtained with the aid of a Technicon autoanalyzer.

and native ribonuclease were prepared in a Technicon autoanalyzer. Series of solutions of each species, in 0.15 M KCl at pH 4.5, were prepared, and 2 ml of each solution was used for the test. The protein was incubated with ninhydrin in the autoanalyzer at 95° for 15 min, and the optical density was read at 570 mμ. Secondly, the optical density of the rest of the above solutions was read at 278 mμ on a Beckman DU spectrophotometer using 1-cm quartz cells.

A plot of optical density of native ribonuclease at 278 mμ *vs.* the optical density of its ninhydrin derivative at 570 mμ was prepared. Assuming that native ribonuclease and the iodinated derivative both have the same extinction coefficient at 570 mμ after reaction with ninhydrin, this plot could be used (in conjunction with the molar extinction coefficient of native ribonuclease) to convert values of the optical density of the ninhydrin derivative of iodinated ribonuclease to concentration. The molar extinction coefficient of the iodinated derivative at 278 mμ was found to be 14,300 ± 200, compared to 9800 for the native protein.

Ultraviolet difference spectra of monoiodotyrosine, diiodotyrosine, and fraction C from iodinated ribonuclease (I_6RNAase C), at pH 11.4 *vs.* 2.1, were measured on a Cary Model 14 recording spectrophotometer, using 1-cm quartz cells, at 25°. These pH values were selected because none of the tyrosines are ionized at the low pH, while they are all in the ionized state at the high pH; also, the buried tyrosines remain un-ionized at these pH values. The difference spectra were unaltered when the reference pH was changed from 2.1 to 4.5.

Spectrophotometric titrations were performed according to the method of Tanford *et al.* (1955). The pH measurements were made at 25° on a Radiometer pH meter, Model No. TTT1a, with an expanded scale, using a combined Radiometer probe electrode (glass calomel), type GK 2021B; the optical density at 295 mμ and 25° was measured on the Beckman DU spectrophotometer.

Performic Acid Oxidation. Performic acid oxidation was carried out according to the method of Hirs (1956). Early experiments in which the iodinated product was fractionated using NaCl–Tris buffer as the eluting agent gave irreproducible analyses for tyrosine. The difficulty was traced to the presence of chloride ion in the performic acid oxidation step. The chloride ion apparently catalyses the destruction of tyrosine by combining with the iodine released from iodotyrosines to form ICl which can iodinate tyrosine (Blatt, 1943). An alternative explanation, suggested by Thompson (1954), is that the chloride impurity, which would be concentrated during lyophlization, could be oxidized to chlorine during the performic acid oxidation. The chlorine would, in turn, chlorinate the uniodinated tyrosine, which would then become susceptible to destruction during the remainder of the oxidation period. In any event, when the NaCl in the eluting buffer system was replaced by sodium acetate, reproducible results were obtained.

After the samples were oxidized and lyophilized they were passed through a Sephadex column (previously equilibrated with 1 M acetic acid) to remove any iodine that may have been liberated.

Enzymatic Digestion. Tryptic (Hirs *et al.*, 1956a), chymotryptic (Hirs *et al.*, 1956b), and peptic (Bailey *et al.*, 1956) digestions were carried out according to the procedures described by Cha and Scheraga (1963). Between 30 and 50 mg of oxidized protein were used.

Amino Acid Analyses. Amino acid analyses (Gundlach *et al.*, 1959) were performed according to the recommendations of Cha and Scheraga (1963), although samples containing a high salt concentration were desalted after they were hydrolyzed. The desalting was done by drying the sample in a Flash-Evaporator, extracting the amino acid into concentrated HCl, and then centrifuging off the salt which is fairly insoluble in concentrated HCl. The HCl was removed by use of the evaporator. Correction factors for destruction of cysteic acid, threonine, serine, and tyrosine were also applied (Rupley and Scheraga, 1963).

The data were interpreted by assuming a theoretical value for the amount of one amino acid, and then calculating the amounts of the others by comparison with that residue. A 10% moisture correction was assumed in the dry weight of the oxidized protein.

Activity of Iodinated Ribonuclease. The spectrophotometric method of Kunitz (1946) was used for measuring the activity of both RNAase and the iodinated derivative in 0.1 M acetate buffer at pH 5.

Results

Fractionation of Iodinated Ribonuclease. The fractionation of the iodinated ribonuclease on the 2.5 × 40 cm preparative column is shown in Figure 1. The relative yield of each fraction, in Figure 1, was estimated from the areas as 6% A, 14% B, 67% C, and 13% D. Fraction C was saved and called I_6RNAase C. *Ca.* 5

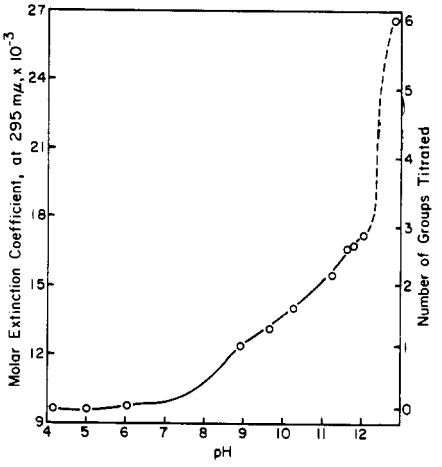

FIGURE 3: Spectrophotometric titration of iodinated ribonuclease at 25°. The solid line represents the reversible portion of the curve while the broken line represents the irreversible part. The broken line was time dependent, and the last point represents a reaction time of 30 min after the pH was brought to 13. The optical density did not increase past the last reading.

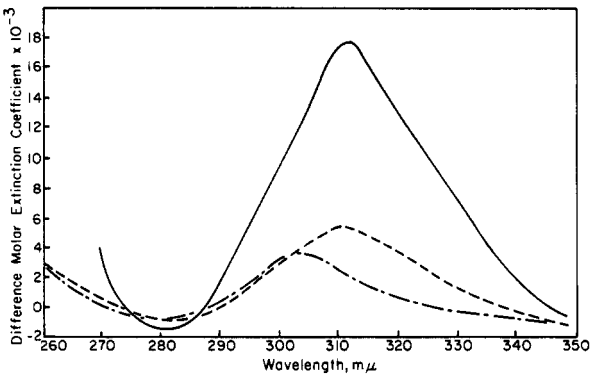

FIGURE 4: Difference spectra of fraction C from iodinated ribonuclease (solid line), 3,5-diiodotyrosine (broken line), and monoiodotyrosine (broken and dotted line). The reference solutions were at pH 2.1, 25°, while the test solutions were at pH 11.4, 25°.

mg of this sample was analyzed on an analytical column, 0.9 × 30 cm, and a single symmetrical peak appeared (Figure 2). The bulk of this sample was assumed to be homogeneous, and it was used in all of the following experiments.

Spectrophotometric Titration of Iodinated Ribonuclease. The spectrophotometric titration of the phenolic hydrogens is shown in Figure 3. The protein ionizes irreversibly and the optical density increases with time above pH 12. Therefore, after the protein solution was brought to pH 13 it was allowed to stand, usually for 30 min, until the optical density remained constant.

From Figure 3 the observed increase in the molar extinction coefficient from pH 12 to 13 was between 8000 and 9000. The value of 2800 for the molar extinction coefficient increase/abnormal tyrosine (Tanford *et al.*, 1955) leads to a result of 2.9–3.2 abnormal residues.

Ultraviolet Difference Spectra. The pH difference spectra of monoiodotyrosine, diiodotyrosine, and iodinated ribonuclease are shown in Figure 4 and Table I. The maximum in the difference spectrum for monoiodotyrosine is at a wavelength of 303 mμ, while that for diiodotyrosine is at 311 mμ (Table I). Mixtures of monoiodotyrosine and diiodotyrosine exhibit spectra with maxima between 303 and 311 mμ (Table I). The difference spectrum for I_6RNAase C shows a maximum at the same wavelength as that for diiodotyrosine. It is also observed that the value of $\Delta\epsilon_{max}$ for I_6RNAase is 17,400 (at 311 mμ) which compares favorably with the value of 16,500, which is three times the $\Delta\epsilon_{max}$ for diiodotyrosine. Thus, the ultraviolet difference spectra indicate that three tyrosines have been converted to diiodotyrosine in the derivative I_6RNAase C. These

TABLE I: Difference Molar Extinction Coefficients of Monoiodotyrosine, Diiodotyrosine, Mixtures of Monoiodo- and Diiodotyrosine, and Iodinated Ribonuclease from the Ultraviolet Difference Spectra at pH 11.4 *vs.* 2 (at 25°).

Sample	$\Delta\epsilon_{max} \times 10^{-3}$	λ_{max} (mμ)
Monoiodotyrosine	3.6	303
Diiodotyrosine	5.5	311
3 Diiodotyrosines	16.5[a]	311
2 Monoiodo- + 1 diiodotyrosine	12.1[a]	307[b]
1 Monoiodo- + 2 diiodotyrosines	14.3[a]	309[b]
1 Monoiodo- + 3 diiodotyrosines	19.8[a]	...
Iodinated RNAase	17.4	311

[a] Obtained by summing appropriate values from monoiodo- and diiodotyrosine at 311 mμ. [b] Measured value, obtained for this mixture.

conclusions do not take into account any possible perturbations from the neighboring charged groups on the model compounds. To a first approximation, this assumption seems valid.

Activity of Iodinated Ribonuclease. The activity of RNAase and I_6RNAase C toward ribonucleic acid is presented in Figure 5. It is observed that 100% activity is retained in the iodinated derivative.

Amino Acid Analyses of Oxidized Iodinated Ribonuclease. The results of the amino acid analyses of several similarly prepared samples of the performic acid oxidized I_6RNAase C are given in Table II. The first column presents the theoretical number of amino acid residues while the other columns contain the experimental results. Performic acid oxidation destroys

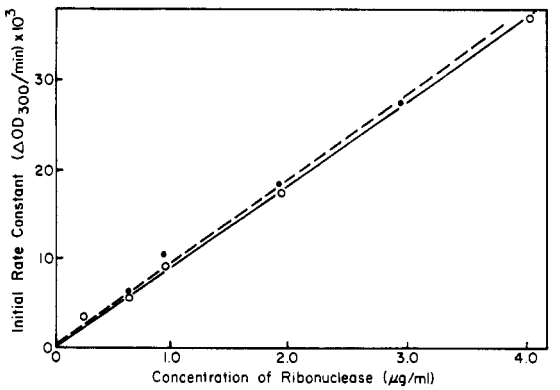

FIGURE 5: Concentration dependence of the initial rate constant for the hydrolysis of ribonucleic acid by ribonuclease (○), and by fraction C from iodinated ribonuclease (●). The concentration of ribonucleic acid was 0.1% in 0.1 M sodium acetate buffer, pH 5.

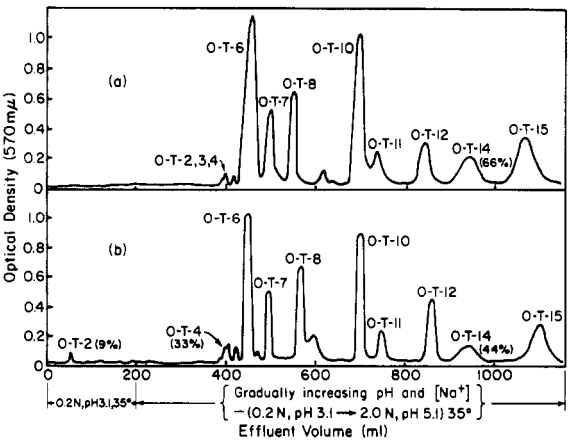

FIGURE 6: Chromatography of peptides from 20 hr of tryptic digest of oxidized protein on a 0.9 × 150 cm column of Dowex 50-X2. The effluent was collected in 2-ml fractions, and the ninhydrin color values were obtained with the aid of a Technicon autoanalyzer. The percentages in parentheses represent the yields of the pertinent peptides; (a) ribonuclease; (b) fraction C.

TABLE II: Amino Acid Analyses of Several Samples (I–III) of Oxidized Iodinated Ribonuclease (Moles of Amino Acid per Mole of Protein).[a]

Amino Acid	Theor	Found		
		I	II	III
Cysteic acid	8	8.40	8.20	7.68
Methionine sulfone	4	3.99	4.05	3.82
Aspartic acid	15	15.00[b]	15.00[b]	15.00[b]
Threonine	10	10.00	10.32	9.84
Serine	15	15.11	15.24	14.91
Glutamic acid	12	11.88	12.04	11.87
Proline	4	3.62	3.80	4.24
Glycine	3	3.25	3.29	3.15
Alanine	12	11.80	12.13	12.57
Valine	9	8.68	8.70	8.96
Isoleucine	3	2.06[c]	2.23[c]	2.48[c]
Leucine	2	2.00	2.12	2.07
Tyrosine	6	2.99	3.04	2.73
Phenylalanine	3	2.86	2.90	2.87
Lysine	10	9.61	10.49	10.16
Histidine	4	3.77	3.79	4.30
Arginine	4	4.01	4.17	4.21

[a] The correction factors for hydrolysis losses (Rupley and Scheraga, 1963): Thr 1.05; Ser 1.12; Cys acid 1.19; Tyr 1.14. [b] Used as reference. [c] Isoleucine is known not to be completely liberated in 22 hr of acid hydrolysis.

all iodinated tyrosines completely (Cha and Scheraga, 1963). The amino acid analyses show that 2.7–3.0 tyrosines remain in oxidized I_6RNAase C, indicating that 3.0–3.3 tyrosines were iodinated. These values compare favorably with the spectrophotometric titration and ultraviolet difference spectra results.

Tryptic Digestion. The peptide elution patterns of the tryptic digest of OxRNAase and OxI_6RNAase C are illustrated in Figure 6. The peptides are numbered in the manner described by Hirs *et al.* (1956a,b) and Bailey *et al.* (1956). The pattern of Figure 6b is similar to those obtained by Cha and Scheraga (1963), except that peptide O-T-14 was present to a slight extent only in one of their two derivatives. Peptide O-T-2 was shifted to an earlier elution time, as previously reported by Cha and Scheraga (1963), and amino acid analysis (Table III) showed that both Tyr 73 and 76 disappeared. Peptide O-T-4 appeared in its normal place in the elution map and, from the amino acid analysis, Tyr 25 was present, as shown in Table III. The low yield is in accord with previous workers (Bailey *et al.*, 1956; Cha and Scheraga, 1963).

Peptide O-T-14 also appeared in its normal position, and the results of the amino acid analysis are presented in Table III. The yield of this peptide from the iodinated species is slightly less than that from uniodinated RNAase. This indicates that some tyrosine may have been iodinated and, upon its destruction during the oxidation procedure, that portion of the peptide shifted to a new elution position. The yield of Tyr 92 and 97 from OxI_6RNAase C with respect to OxRNAase is also slightly lower. This is also evidence that some tyrosine may have been destroyed.

Chymotryptic Digestion. The peptide elution patterns for both the chymotryptic digests of OxRNAase and OxI_6RNAase C were identical with the ones presented by Cha and Scheraga (1963). Peptides O-C-3 and O-C-29 (both containing Tyr 115) and peptide O-C-30 (adjacent to O-C-3) all disappeared from the chromatographic pattern of OxI_6RNAase C. They were observed in the OxRNAase pattern. The disappearance of these peptides indicates that Tyr 115 was destroyed (iodi-

TABLE III: Amino Acid Analysis of O-T-2, O-T-4, and O-T-14 Obtained from a Tryptic Digest of Oxidized Iodinated Ribonuclease, and O-T-14 Obtained from a Tryptic Digest of Oxidized Ribonuclease (Moles of Amino Acid per Mole of Peptide).

	O-T-2 (Residues 67–85)		O-T-4 (Residues 11–31)		O-T-14 (Residues 92–98)		
Amino Acid	Theor	I_6RNAase	Theor	I_6RNAase[b]	Theor	RNAase A	I_6RNAase
Cysteic acid	2	2.24	1	1.29	1	1.06	0.91
Methionine sulfone	1	0.89	3	2.63			
Aspartic acid	3	2.95	3	3.38	1	0.89	1.03
Threonine	3	3.00[a]	1	1.39			
Serine	3	3.19	6	6.40		0.11	0.10
Glutamic acid	2	1.90	2	2.30			
Proline					1	1.11	0.90
Glycine	1	1.26		0.33		0.10	
Alanine		0.36	2	2.08	1	1.00[a]	1.00[a]
Valine						0.12	
Isoleucine	1	1.01		0.29			
Leucine							
Tyrosine	2	Disappeared	1	0.55[c]	2	1.90	1.71
Phenylalanine							
Lysine			1	1.16		0.90	0.84
Histidine			1	1.00[a]			
Arginine	1	1.03					
Yield of peptide (%)		9		33		66	44

[a] Assumed as reference. [b] Probably slightly contaminated with O-T-2. [c] Low result in accord with Cha and Scheraga (1963) and Bailey *et al.* (1956).

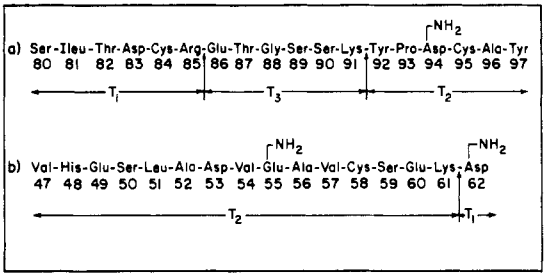

FIGURE 7: Amino acid sequence of peptides (a) O-C-14 and (b) O-C-15 obtained from the enzymatic digestion by chymotrypsin. The positions where trypsin cleaves each of the peptides are indicated by the perpendicular arrows, and the tryptic peptides which are obtained are indicated by the horizontal arrows.

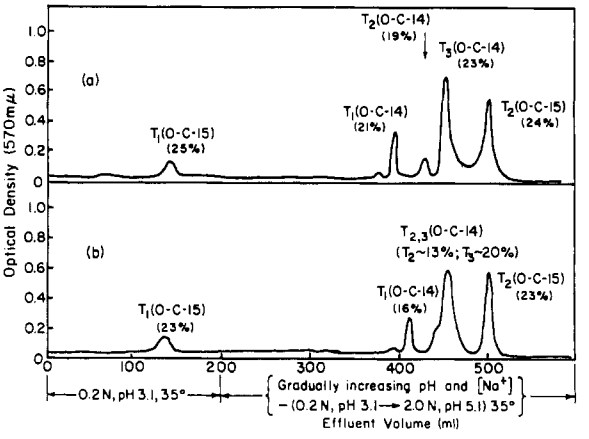

FIGURE 8: Chromatography of peptides from 20 hr of tryptic digest of a mixture of peptides O-C-14 and O-C-15. The chromatographic conditions are the same as in Figure 6. The figures in brackets represent the yields of each peptide; (a) ribonuclease; (b) fraction C.

nated), and that cleavage at the Tyr–Val, 115–116, bond did not occur.

Peptides O-C-11 and O-C-12 from both the iodinated and uniodinated protein derivatives were studied. The results were similar to those obtained by Cha and Scheraga (1963). Analysis of the peptide showed that O-C-12 containing Tyr 73 disappeared completely while O-C-11 containing Tyr 25 was present.

Initial examination of the Dowex 50-X2 chromatogram showed that peptides O-C-14 and O-C-15 were present; however, it was difficult to determine correctly whether O-C-14 contained one or two tyrosine residues because of the large number of residues in each

TABLE IV: Amino Acid Analysis Obtained from a Tryptic Digest of O-C-14 and O-C-15, Previously Obtained from a Chymotryptic Digest of Oxidized Iodinated Ribonuclease (Moles of Amino Acid per Mole of Peptide).

	T_1(O-C-14)[a] (Residues 80–85)			T_2(O-C-14)[b] (Residues 92–97)			T_3(O-C-14)[c] (Residues 86–91)			T_1(O-C-15) (Residue 62)			T_2(O-C-15) (Residues 47–61)		
Amino Acid	Theor	RNAase	I_6RNAase	Theor	RNAase	I_6RNAase	Theor	RNAase	I_6RNAase	Theor	RNAase	I_6RNAase	Theor	RNAase	I_6RNAase
Cysteic acid	1	1.10	1.06	1	1.02	0.92							1	1.14	1.05
Methionine sulfone															
Aspartic acid	1	0.75	1.04	1	1.00[c]	1.00[c]				1	1	1	1	1.11	1.07
Threonine	1	0.75	0.99				1	0.98	0.97						
Serine	1	1.00[c]	1.00[c]				2	1.97	2.07				2	1.98	2.06
Glutamic acid							1	1.20	1.09				3	2.85	3.02
Proline				1	0.89	0.90									
Glycine			0.11				1	1.00[c]	1.00[c]						
Alanine			0.17	1	0.86	0.97							2	1.88	2.10
Valine			0.13										3	3.24	2.93
Isoleucine	1	0.69	0.89												
Leucine													1	1.00[c]	1.00[c]
Tyrosine				2	1.58	1.49									
Phenylalanine															
Lysine			0.11										1	1.00	1.02
Histidine													1	0.88	0.96
Arginine	1	0.80	0.93				1	1.11	0.97						
Yield of peptide (%)		21	16		19	13		23	20		25	23		24	23

[a] Tryptic notation as peptide appeared on elution pattern. [b] The amino acid analyses were performed on the mixture of T_2(O-C-14) and T_3(O-C-14) in the case of I_6RNAase, but on the separated peptides in the case of RNAase. No amino acid is common to both peptides. [c] Assumed as reference.

peptide. The chymotryptic digestion was then repeated and the mixture of peptides O-C-14 and O-C-15 was isolated and then digested a second time, however, this time with trypsin. The tryptic digestion scheme for O-C-14 and O-C-15 is illustrated in Figure 7, and the elution pattern in Figure 8. The amino acid analyses for both OxRNAase and OxI_6RNAase C are given in Table IV. It can be seen that the yield of the two tryptic peptides, T_1 and T_2, from peptide O-C-15 is essentially the same for both OxRNAase and OxI_6RNAase C. This was to be expected as none of these amino acids had been modified during the iodination. However, the yields of the three tryptic peptides from O-C-14 were slightly lower by 15–30% in the iodinated species. This meant that some of either Tyr 92 or 97 had been iodinated. The tyrosine content in T_2 (O-C-14) of both species was somewhat lower than the theoretical value of two. The reason for this is not understood; however, the relative amounts of tyrosine in both the iodinated and uniodinated protein were about the same.

Peptic Digestion. The results from the peptic digest were also similar to those of Cha and Scheraga (1963). Peptide O-P-5 disappeared from the elution pattern of I_6RNAase C, indicating that Tyr 115 had been iodinated, and the peptide had moved to a new position in the pattern. Attempts at location of this modified peptide were unsuccessful. The amino acid analysis of O-P-5 from OxRNAase is presented in Table V. The low yield is in accord with those of previous workers (Bailey *et al.*, 1956; Cha and Scheraga, 1963).

TABLE V: Amino Acid Analysis of O-P-5 Obtained from the Peptic Digest of Oxidized Ribonuclease and Oxidized Iodinated Ribonuclease (Moles of Amino Acid per Mole of Peptide).

	O-P-5 (Residues 109–120)		
Amino Acid	Theor	RNAase	I_6RNAase
Cysteic acid	1	1.33	
Methionine sulfone			
Aspartic acid	1	1.17	Peptide disappeared
Threonine			
Serine			
Glutamic acid	1	0.95	
Proline	2	2.18	
Glycine	1	1.05	
Alanine	1	1.00[a]	
Valine	2	1.86	
Isoleucine			
Leucine			
Tyrosine	1	0.40[b]	
Phenylalanine	1	0.83	
Lysine			
Histidine	1	0.82	
Arginine			
Yield of peptide (%)		31	

[a] Assumed as reference. [b] Low value of tyrosine in accord with Bailey *et al.* (1956) and Cha and Scheraga (1963).

Discussion

Earlier workers (Cha and Scheraga, 1963; Donovan, 1963) used the iodination conditions recommended by Hughes and Straessle (1952). Hughes and Straessle concluded that iodination of tyrosine was more specific at higher pH values and recommended at pH of 9.5. However, in the case of RNAase, two of the amino acids most susceptible to oxidation, cysteine and tryptophan, are absent. Furthermore, the four methionine residues are apparently buried (Gundlach *et al.*, 1959). In our study, at pH 6.7, we found no evidence of oxidative side reactions.

Since there has been no thorough study of the conformation of RNAase above pH 7, it is possible that the conformation of RNAase at pH 9.5 may differ from that at pH 7. Indeed, we find that the third buried tyrosine is not iodinated at neutral pH, while Cha and Scheraga (1963) obtained a derivative with four iodinated tyrosines, and Donovan's (1963) derivative may have had a fourth tyrosine at least partially iodinated.

Although the presence of four components from the carboxymethylcellulose chromatography may indicate different degrees of iodination, only the main component was studied because it contained the major part of the material. From the spectrophotometric titration, ultraviolet difference spectra, and amino acid analysis, it appears as if three tyrosines have been iodinated. It may also be concluded that the three tyrosines were diiodinated, on the basis of a comparison of the iodinated protein with several model compounds. The calculated difference in molar extinction coefficients of 19,800 for three diiodotyrosines plus one monoiodotyrosine would seem to be too high while the value of 14,300 for two diiodotyrosines plus one monoiodotyrosine would seem to be too low. Secondly, the maximum wavelength of 311 mμ corresponds to pure diiodotyrosine rather than to a mixture of mono- and diiodotyrosines.

Enzymatic digestion of oxidized iodinated ribonuclease provides evidence to show which are the normal and abnormal tyrosines. It had been previously shown (Cha and Scheraga, 1963) that Tyr 25 was not iodinated in the native state, and even remained buried when the RNAase molecule was exposed to partial proteolytic digestion (Sela and Anfinsen, 1957; Fujioka and Scheraga, 1965). These results have been corroborated in this work by obtaining O-T-4 from tryptic digestion and O-C-11 from chymotryptic digestion, both of which contain Tyr 25.

Tyr 73 and 76 disappeared upon iodination as was previously reported by Cha and Scheraga (1963). Peptide O-T-2 shifted its position on the elution pattern, and both tyrosines disappeared completely in the amino acid analysis of this peptide. Further evidence for the

iodination of Tyr 73 was given by the disappearance of peptide O-C-12 containing this residue. Attempts to find this peptide failed.

Evidence has been presented for the iodination of Tyr 115 in both the peptic and chymotryptic digests. Peptides O-P-5, O-C-3, O-C-29, and O-C-30 all disappeared. This had also been observed by Cha and Scheraga (1963). Efforts to locate these peptides elsewhere in the elution pattern also failed.

The presence of uniodinated residues Tyr 92 and 97 has been supported by two pieces of evidence in this work. Peptide O-T-14, from the tryptic digest, was located at its normal position and both tyrosines were found to be present in the amino acid analysis. A slightly lower yield for the peptide and for the number of tyrosyl residues in the uniodinated derivative suggests that some of the tyrosine may have been iodinated. Donovan (1963) and Cha and Scheraga (1963) both found Tyr 92 iodinated and Tyr 97 buried. This leads to the conclusion that, under the conditions of iodination given in this work, Tyr 92 was less exposed to iodination. The lower yield of peptide and number of tyrosine residues may reflect a *partial* burial of Tyr 92.

Tryptic digestion of O-C-14 and O-C-15 was the second supporting piece of evidence which showed that Tyr 92 and 97 were largely uniodinated. Although peptides O-C-14 and O-C-15 could not be separated by chromatography on Dowex 50-X2 the presence of both peptides in the peptide chromatogram suggested little if any modification of the tyrosines. From the tryptic digestion of the mixture of O-C-14 and O-C-15, it was found that the peptide T_2 (O-C-14), containing Tyr 92 and 97, was present in a 70% yield compared to the yield from the uniodinated derivative. The other two peptides, T_1 and T_3, from O-C-14 were also lower in yield compared to RNAase. Peptides T_1 and T_2 from O-C-15 had almost the same yield in both native RNAase and the iodinated derivative. The lower yields in the tryptic peptides from O-C-14 suggest a partial iodination of Tyr 92.

It appears that the three exposed tyrosines are 73, 76, and 115 under the conditions of iodination, while the three abnormal tyrosines are 25, 92 and 97. From previous difference spectra studies (Bigelow, 1961; Hermans and Scheraga, 1961) it appears that one of the tyrosines may be less abnormal than the other two. This work, along with that of Cha and Scheraga (1963), seems to confirm that possibility, and the *partially* abnormal tyrosine is most likely 92. This conclusion was drawn from the fact that two of the three tyrosines (Tyr 25 and 97) were found buried upon iodination at pH 9.5 (references previously cited) and only under the present milder conditions was Tyr 92, along with Tyr 97 and 25, found to be buried.

Structural Implications. With the location of the third buried tyrosine (Tyr 92), it is now possible to identify the three tyrosyl–carboxyl interactions. These are discussed in the accompanying paper (Li *et al.*, 1966).

Acknowledgment

The technical assistance of Mrs. Bonnie Dalzell with the amino acid analyses is gratefully acknowledged.

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