Structural Studies of Ribonuclease. XII. Enzymic Hydrolysis of Active Tryptic Modifications of Ribonuclease*

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Enzymically active derivatives of ribonuclease were previously obtained by digestion with trypsin at 60°, followed by fractionation on an IRC-50 column. These derivatives had one peptide bond split, either lys 31–ser 32 or arg 33–asp(NH_2) 34, the mixture of these two derivatives having previously been designated component IV. In the present work, further digestion of component IV *at room temperature* yielded eight components which were fractionated on an IRC-50 column. These components are designated 1 to 8. The amino acid composition and N-terminal and C-terminal groups of these components have been determined. Components 2, 3, and 4 are small peptides, whereas components 1, 5, 6, 7, and 8 have high molecular weight; component 8 is unreacted starting material (i.e., component IV), component 7 (previously designated component III) is missing the ser 32–arg 33 dipeptide, component 6 is missing the tetrapeptide asp(NH_2) 34–lys 37, and component 5 is missing the hexapeptide ser 32–lys 37. The other components have also been identified. Since prolonged digestion did not provide other products, it appears that five possible peptide-bond sites of tryptic attack of component IV are not hydrolyzed, suggesting that these five positions are less exposed than the others at room temperature. Also, chymotrypsin did not attack components IV or III at room temperature. Some of the new components still possess limited enzymic activity. These results, together with the determined sequence of events in the cleavage process, provide information about the folding of the ribonuclease molecule in solution.

The proteolytic enzymes trypsin and chymotrypsin do not attack native ribonuclease at room temperature. However, ribonuclease is digested by these enzymes at elevated temperatures, where the substrate undergoes a thermal transition, giving intermediates which can be separated on an IRC-50 column. The intermediate components from the chymotryptic digestion are enzymically inactive, whereas those from the tryptic digestion have some activity (Rupley and Scheraga, 1963; Ooi *et al.*, 1963). Two of the active intermediates from the tryptic digestion of ribonuclease at 60° were designated components III and IV (Ooi *et al.*, 1963). Component IV was a mixture of two species, each having one peptide bond split, lys 31–ser 32 and arg 33–asp(NH_2) 34, respectively; component III had both these peptide bonds split and therefore lacked the ser 32–arg 33 dipeptide. Since component IV could be obtained in relatively high yield, it was of interest to investigate the further action of trypsin and chymotrypsin on it in order to determine how component IV differed in its conformation (specifically, in its resistance to enzymic hydrolysis) from native ribonuclease. It was expected that further tryptic digestion of component IV would lead to component III, as well as to other products.

In contrast to the behavior of native ribonuclease, component IV is digestible by trypsin *at room temperature*. The rate of digestion is, of course, considerably higher at elevated temperatures (e.g., 40°), where component IV undergoes a thermal transition (Ooi and Scheraga, 1964). The new components, obtained from the tryptic digestion of component IV at room temperature, provide information about the folding of the ribonuclease molecule in solution, and about the relationship between the structure and the enzymic activity of this protein.

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EXPERIMENTAL

Materials.—Essentially all the materials used in this investigation have been described previously (Rupley and Scheraga, 1963; Ooi *et al.*, 1963). Chymotrypsin was purchased from Worthington. All other chemicals were analytical reagent grade where possible.

Component IV was prepared by digestion of ribonuclease A with trypsin at 60°, fractionated on an IRC-50 column with potassium phosphate buffer, desalted by gel filtration, and lyophilized, as described previously (Ooi *et al.*, 1963). Rechromatography on IRC-50 improved the state of purity of component IV.

Method.—Digestion of component IV with trypsin was carried out in a *p*H stat (Radiometer TTT1) at constant temperature, recording the base uptake to maintain a constant *p*H (Rupley and Scheraga, 1963). The lyophilized component IV was dissolved in 0.01 M KCl, *p*H 7.9, at concentrations of 1–10 mg/ml, and the temperature was varied from 21 to 45°. The reaction was stopped by lowering the *p*H to about 5 by addition of 0.1 M, *p*H 4.0 sodium acetate buffer, and by addition of diisopropylfluorophosphate to about ten times excess of trypsin. The solution was then cooled quickly to 0° (and lyophilized in preparative-scale experiments). The same procedure was used when chymotrypsin was substituted for trypsin.

IRC-50 chromatographic analyses of digestion mixtures were performed using columns of Amberlite IRC-50, XE-64 resin (sieved 200 mesh, wet) in conjunction with a Technicon Autoanalyzer, as described by Rupley and Scheraga (1963). The column sizes were 1 × 30 cm for analytical-scale experiments and 1.7 × 40 cm for preparative-scale work. The eluting buffer was controlled with the aid of four chambers of a Technicon Auto-grad, the first three chambers containing 0.15 M sodium phosphate, 1 mM versenate buffer, *p*H 6.40, and the last chamber containing 1 M sodium phosphate, 1 mM versenate buffer, *p*H 6.47. In the analytical runs, the chromatogram was obtained with a Technicon Autoanalyzer to develop and record the ninhydrin color at a flow rate of about 0.35 ml/min. In the preparative-scale experiments

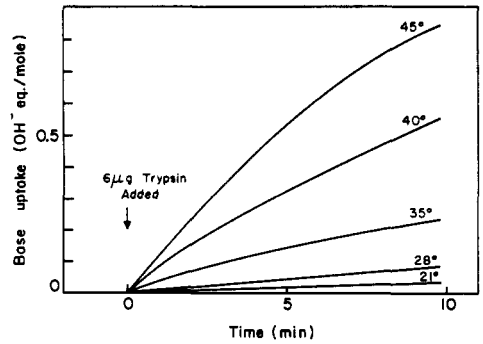

FIG. 1.—Digestion of component IV with trypsin over a 10-minute interval at various temperatures. The initial concentration of component IV was 4 mg/4 ml in 0.01 M KCl at *p*H 7.9. The amount of trypsin (6 μg) is indicated in the figure.

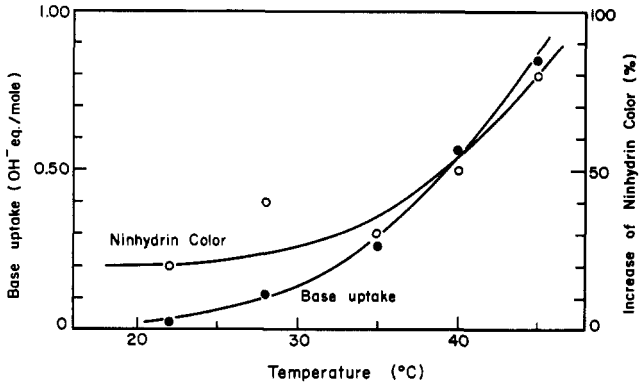

FIG. 2.—Temperature dependence of the rate of digestion expressed in terms of the base uptake (●) and the increase of ninhydrin color (○) after 10 minutes. The data on base uptake were obtained from Fig. 1.

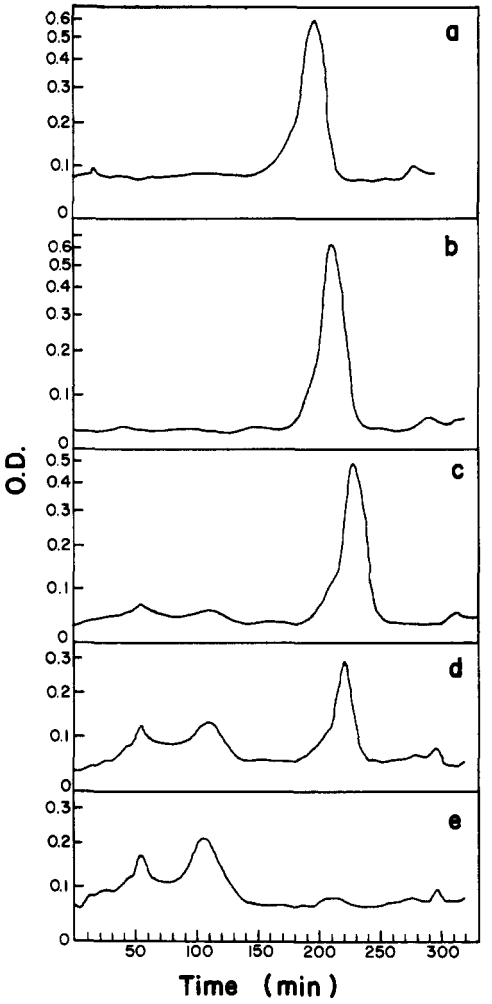

FIG. 3.—Chromatograms of tryptic digestion mixtures of component IV on IRC-50. The digestions were performed for 10 minutes at: (a) 21°, (b) 28°, (c) 35°, (d) 40°, and (e) 45°. The ordinate represents the ninhydrin color value, and the abscissa is the effluent volume expressed on a time scale on which 0.35 ml corresponds to 1 minute.

the effluent was collected with a fraction collector and the optical density of each tube was measured at 278 mμ with a Beckman DU spectrophotometer. Occasionally the ninhydrin color of each tube was developed and recorded with a Technicon sampler device.

Ribonuclease activity against RNA was measured as described previously (Ooi *et al.*, 1963).

Gel filtration was performed on a 2.4 × 40–cm column of Sephadex G-25, the phosphate buffer being replaced by 0.05 M ammonium acetate, followed by repeated lyophilization (Ooi *et al.*, 1963).

The procedures described by Rupley and Scheraga (1963) and by Ooi *et al.*, (1963) were used for amino acid analyses of protein hydrolysates, and N-terminal and C-terminal group analyses. Identification of DNP-asp, DNP-glu, and DNP-his was accomplished by paper chromatography with a tertiary amyl alcohol system (Blackburn and Lowther, 1951). DNP-asp and DNP-glu were eluted from the spot after paper chromatography with a toluene system, the water phase of the mixture of DNP-amino acids being subsequently extracted with ethyl acetate to obtain DNP-his.[1]

RESULTS

Temperature Dependence of Digestion.—In order to determine the proper conditions under which to obtain high-molecular-weight intermediates from the tryptic digestion of component IV, the digestion was performed for a fixed time interval (10 minutes) at various temperatures. Figure 1 shows the rate of digestion, expressed as base uptake in equivalents per mole of ribonuclease; Figure 2 shows the temperature dependence of the base uptake and increase of ninhydrin color. The curves of Figure 2 seem to reflect the thermal behavior of component IV; i.e., the slow digestion below 40° and the rapid one above this temperature are the expected behavior since the molecule is thermally unfolded above 40° (Ooi and Scheraga, 1964).

The chromatograms of reaction mixtures from tryptic digestions for 10 minutes at various temperatures are shown in Figure 3. At low temperature only one main peak appears, at the position of unreacted component IV; however, the asymmetry indicates that some digested material elutes as a shoulder ahead of the peak corresponding to the main component. This means that trypsin attacks component IV at room temperature, but at a slow rate, as already seen in Figure 1. With increasing temperature the relative height of the main peak decreases and new peaks appear in the earlier parts of the chromatogram. However, component III of the earlier paper (Ooi *et al.*, 1963), i.e., the one missing the ser 32–arg 33 dipeptide, which was obtained by digestion of ribonuclease A with trypsin at 60°, did not appear in

[1] Abbreviations used in this work: DNP, dinitrophenyl; FDNB, 1-fluoro-2,4-dinitrobenzene.

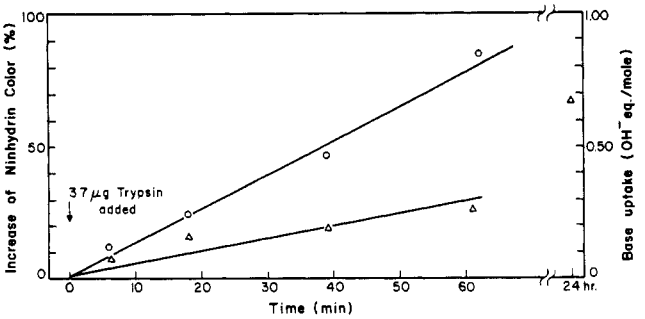

FIG. 4.—Time course of the tryptic digestion of component IV at room temperature, expressed in terms of increase of ninhydrin color (△) and base uptake (○). The initial concentration of component IV was 18 mg/18 ml in 0.01 M KCl at *p*H 7.9.

the high-temperature digests of component IV. Its failure to appear is probably due to the ease of digestion of component III at elevated temperatures. The asymmetric and rather broad shapes of the newly produced peaks in Figure 3e indicate that these components are quite heterogeneous. Therefore, since it would be very difficult to isolate pure high-molecular-weight derivatives from the high-temperature digestion mixtures, it was decided to carry out the digestion at room temperature.

Digestion at Room Temperature.—The time course of the tryptic digestion of component IV at room temperature, in terms of increase of ninhydrin color and base uptake, is shown in Figure 4. After 24 hours, when digestion does not seem to proceed any further, the increase of ninhydrin color reaches 68%, which corresponds to an average of about five or six bonds cleaved, while the theoretical number of bonds in component IV which can be attacked by trypsin is eleven. Therefore some parts of the molecule of component IV are sufficiently folded to prevent attack by trypsin, even though several of the products of digestion of component IV at room temperature have a somewhat looser structure (Ooi and Scheraga, 1964).

The IRC-50 chromatograms of the digested solutions at various stages of reaction are shown in Figure 5. As digestion proceeds, the main peak (unreacted component IV) decreases in size and new peaks appear; after 24 hours the main peak has essentially disappeared. The peaks in Figure 5b and c may be designated 1–8. In a separate experiment, the digestion mixture corresponding to Figure 5b was run on Sephadex G-75 and the only material which was collected was that which eluted at the front. After lyophilization this fraction was rerun on an IRC-50 column, and components 1, 5, 6, 7, and 8 were found in the chromatographic pattern; these components were thus identified as high-molecular-weight material. The details of amino acid and end-group analyses of the material corresponding to these peaks will be described.

Separation of Each Component.—In order to obtain sufficient material for identification of the various components of Figure 5, large-scale preparative runs were carried out. A typical chromatogram from such an experiment is shown in Figure 6. The degree of digestion of the mixture of Figure 6 corresponds to that of Figure 5b. It can be seen that each effluent peak, except that of component 2 which is a shoulder on peak 3, could be resolved and separated by combining the appropriate tubes near the maximum of each peak. Component 2 was obtained from a 60-minute digestion, corresponding to the degree of digestion shown in Figure 5c.

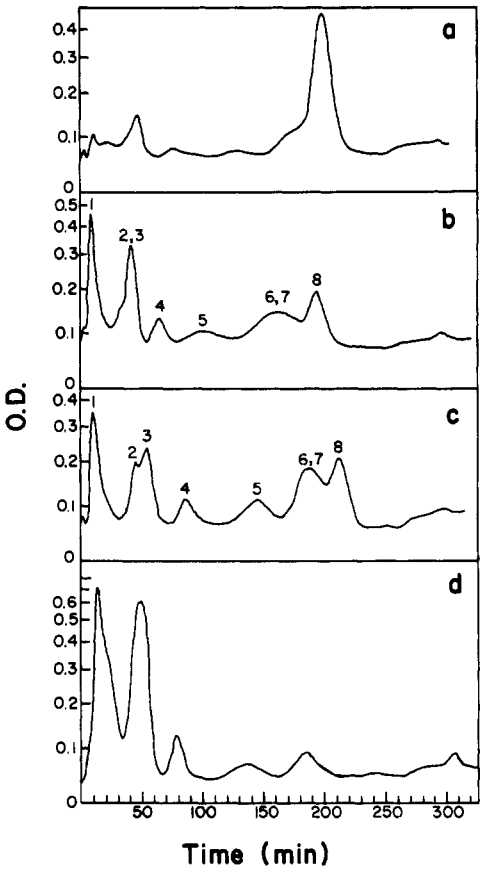

FIG. 5.—Chromatograms of tryptic digestion mixtures of component IV on IRC-50. The digestions were performed at room temperature for: (a) 6 minutes, (b) 38 minutes, (c) 60 minutes, and (d) 24 hours. The ordinate and abscissa are the same as in Fig. 3.

It is interesting to note that component 5, which has some activity, elutes at a position close to where ribonuclease would *if it were present.* However, the activity of component 5 cannot be due to contamination of the starting material by ribonuclease, since the activity of component 5 increases as the amount of component 5 increases when digestion is carried out for various lengths of time. Therefore we may conclude from Figure 6 that components 1–4 are inactive and that components 5–8 are active to various degrees.

Preliminary estimates of the molecular size of the new components by gel filtration indicated that components 1, 5, 6, 7, and 8 were of high molecular weight.

Several tubes near the maximum of those peaks of Figure 6 corresponding to high-molecular-weight material (peaks 1, 5, 6, 7, and 8) were rerun on IRC-50 after desalting by gel filtration on Sephadex G-25 and repeated lyophilization to remove volatile salt. The resulting chromatograms are shown in Figure 7. It can be seen that the various cuts from Figure 6 appear almost as single peaks in Figure 7. However, the separation is not completely sharp, leading to asymmetries in the peaks of Figure 7. Nevertheless, because of the low yields of the partially purified components of Figure 7, they were used for analysis without further purification. It can be seen from the results obtained below that the small amounts of impurities did not prevent a proper identification to be made of the major components corresponding to each peak.

Identification of Cleavage Positions; Amino Acid Analyses.—The amino acid composition of each component is shown in Tables I and II. The "theoretical"

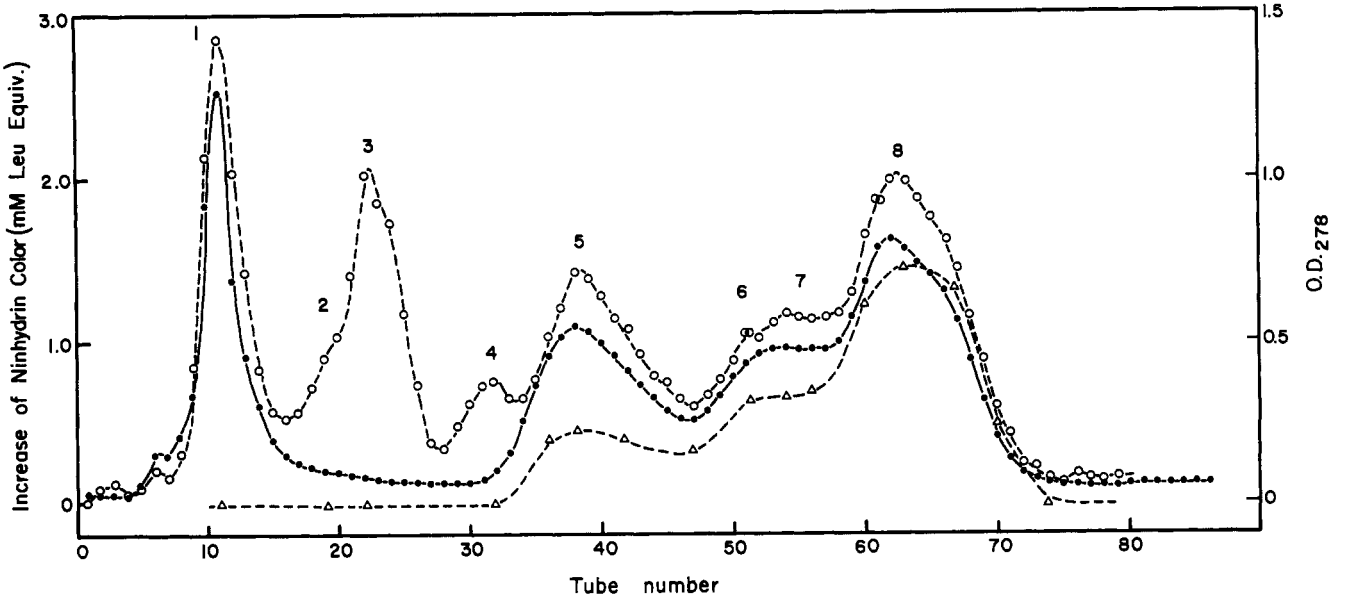

FIG. 6.—Chromatogram of a preparative-scale tryptic-digestion mixture of component IV on IRC-50, expressed in terms of increase of ninhydrin color (○), optical density at 278 mμ (●), and enzymic activity in arbitrary units on the OD_{278} scale (△). The digestion was carried out with 200 mg of component IV and 1.3 mg of trypsin in 20 ml of 0.01 M KCl, *p*H 7.8, for 40 minutes.

TABLE I
AMINO ACID COMPOSITION OF LARGE COMPONENTS[a]

	1		5		6		7		8	
	Theor	Expt	Theor	Expt	Theor	Expt	Theor	Expt	Theor	Expt
Aspartic acid	13	12.9	14	14.1	14	13.8	15	15.0	15	15.0
Threonine	6	5.9	9	8.9	9	9.3	10	9.8	10	10.5
Serine	14	14.2	14	14.2	15	14.5	14	14.3	15	15.2
Glutamic acid	9	8.8	12	12.3	12	11.6	12	12.2	12	11.9
Proline	4	4.3	4	4.6	4	4.3	4	4.4	4	4.4
Glycine	3	2.7	3	3.2	3	3.1	3	3.0	3	3.2
Alanine	8	8.2	12	11.6	12	11.6	12	12.1	12	12.3
Half-cystine	8	7.7	8	7.9	8	8.6	8	8.5	8	7.9
Valine	9	9.0	9	8.6	9	8.6	9	8.8	9	8.5
Methionine	4	3.9	4	3.8	4	3.9	4	3.7	4	3.7
Isoleucine	3	2.4	3	2.1	3	2.0	3	2.4	3	2.2
Leucine	1	1.1	1	1.2	1	1.4	2	1.8	2	2.0
Tyrosine	6	5.6	6	5.7	6	6.2	6	6.1	6	6.3
Phenylalanine	2	2.3	3	3.2	3	3.0	3	3.0	3	3.2
Lysine	6	5.7	9	9.1	9	9.5	10	9.9	10	10.7
Histidine	4	4.0	4	3.9	4	3.9	4	4.0	4	4.2
Arginine	2	1.7	3	3.1	4	3.7	3	3.3	4	4.0

[a] Given in moles of amino acid/mole of protein. The correction factors used in the calculations are the same as those used by Rupley and Scheraga (1963).

values in Table I are the nearest whole numbers to the experimental values. Compared to native ribonuclease, component 1 lacks 2 aspartic acid, 4 threonine, 1 serine, 3 glutamic acid, 4 alanine, 1 leucine, 1 phenylalanine, 4 lysine, and 2 arginine residues. These values indicate that the following peptides (Hirs *et al.*, 1960; Smyth *et al.*, 1963) have been lost to produce component 1 from ribonuclease: lys 1–arg 10, ser 32–lys 37, and thr 99–lys 104. Component 5 lacks 1 aspartic acid, 1 threonine, 1 serine, 1 leucine, 1 lysine, and 1 arginine residue, i.e., it lacks the hexapeptide ser 32—lys 37. Component 6 lacks 1 aspartic acid, 1 threonine, 1 leucine, and 1 lysine residue, i.e., it lacks the tetrapeptide asp(NH_2) 34–lys 37. Component 7 lacks 1 serine and 1 arginine residue, i.e., it lacks the dipeptide ser 32–arg 33 previously (Ooi *et al.*, 1963) designated component III. Component 8 has the same amino acid composition as that of ribonuclease A, suggesting that it is unreacted component IV. These results are summarized in Table III.

The amino acid composition data of Table II indicate that components 2, 3, and 4 are small peptides released during the digestion; the low values of OD_{278} for these components (see Fig. 6) are consistent with this conclusion. Apparently component 4 is the tetrapeptide asp(NH_2) 34–lys 37. Component 3 appears to be a mixture of 0.14 μmole of the peptide lys 1–lys 7 and 0.17 μmole of the peptide thr 99–lys 104. The results for component 2 show that the main amino acids in this mixture are aspartic acid, glutamic acid, phenylalanine, and arginine, indicating that it contains the tripeptide phe 8–glu 9–arg 10, since this is the only small phenylalanine-containing peptide which is obtained by tryptic digestion. The appearance of aspartic acid is probably due to the contamination of this material by other peptides. Although it is possible to estimate a composition of component 2 by assuming that this component is a mixture of several peptides so as to fit the experimental data for each amino acid with theoretical ones calculated by summation over

TABLE II
AMINO ACID COMPOSITION OF SMALL PEPTIDES[a]

	2	3 (0.14 μmole 1–7 0.17 μmole 99–104)		4 (0.37 μmole 34–37)	
	Expt	Theor	Expt	Theor	Expt
Aspartic acid	0.635	0.17	0.19	0.37	0.37
Threonine	0.183	0.48	0.46	0.37	0.36
Serine	0.322				
Glutamic acid	0.650	0.31	0.35		
Proline	0.115				
Glycine	0.074				
Alanine	0.216	0.59	0.60		
Half-cystine	0.066				
Valine	0.198				
Methionine	0.064				
Isoleucine	0.049				
Leucine	0.042			0.37	0.33
Tyrosine	0.113				
Phenylalanine	0.475				
Lysine	0.191	0.45	0.46	0.37	0.37
Histidine	0.093				
Arginine	0.715				

[a] The correction factors used in the calculations are the same as those used by Rupley and Scheraga (1963). The compositions are those which were assumed in order to make the theoretical values agree with the experimental ones.

TABLE III
IDENTIFICATION OF THE COMPONENTS OF TABLE I

Component	Bonds Split[a]	Amino Acids Missing	Previous Identification[b]
1	arg 10–glu(NH_2) 11, lys 31–ser 32, lys 37–asp 38, lys 98–thr 99, lys 104–his 105	lys 1–arg 10, ser 32–lys 37, thr 99–lys 104	
5	lys 31–ser 32, lys 37–asp 38	ser 32–lys 37	
6	arg 33–asp(NH_2) 34, lys 37–asp 38	asp(NH_2) 34–lys 37	
7	lys 31–ser 32, arg 33–asp(NH_2) 34	ser 32–arg 33	III
8	Mixture of two species, lys 31–ser 32 and arg 33–asp(NH_2) 34, respectively	None	IV

[a] An additional cleavage at lys 7–phe 8, required for the liberation of the tripeptide phe 8–arg 10, was also observed (see Table II). [b] Ooi *et al.* (1963).

the assumed peptides, a result derived in this way would seem to be rather arbitrary. The results shown in Table II indicate that components 2, 3, and 4 are small peptides, as would be expected from the data on the large components of Table I.

The amino acid composition data of Tables I and II, summarized in part in Table III, indicate that the following bonds of ribonuclease are split in the formation of component IV (or component 8) at 60° and in its further digestion at room temperature: arg 10–glu(NH_2) 11, lys 31–ser 32, arg 33–asp(NH_2) 34, lys 37–asp 38, lys 98–thr 99, and lys 104–his 105. From the data of Table II, it is quite likely that the lys 7–phe 8 bond is also split.

Amino acid composition data, by themselves, are not sufficient to locate all the split peptide bonds since

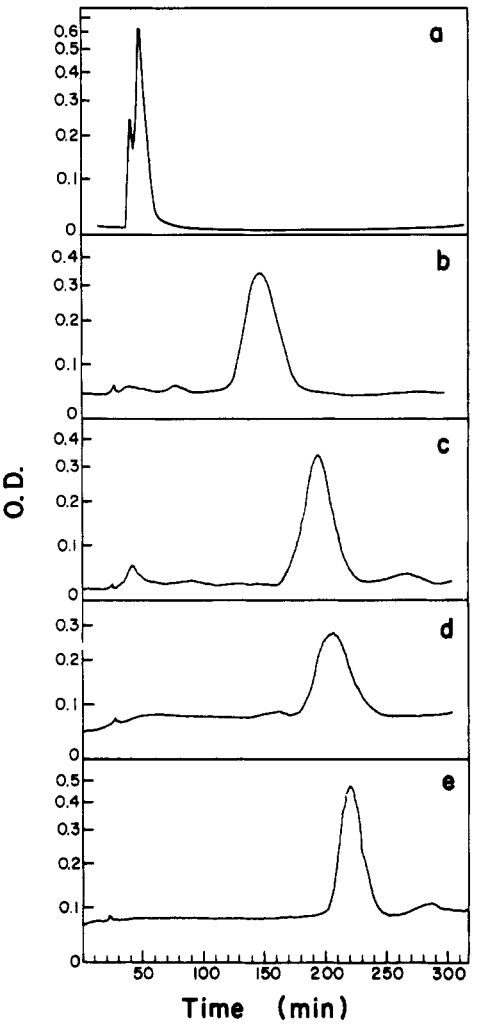

FIG. 7.—Rechromatography on IRC-50 of the components from Fig. 6. The ordinate and abscissa are the same as in Fig. 3. a, b, c, d, e, represent components 1, 5, 6, 7, 8, respectively.

TABLE IV
N-TERMINAL GROUP ANALYSES[a]

	1	5	6	7	8
Aspartic acid	0.79	0.90	0.81	0.74	0.40
Serine					0.45
Glutamic acid	0.57	Trace			
Lysine		0.95	0.96	0.96	0.95
Histidine	0.22[b]				

[a] In moles of DNP-amino acid per mole of component. [b] Not corrected for hydrolysis and chromatographic losses.

such bonds could be split without liberation of amino acids. Therefore end-group analyses were also carried out to confirm the above conclusions and also to locate single splits, if any such occur.

N-Terminal Group Analyses.—The N-terminal residues of the large components were determined by the FDNB method, and are listed in Table IV. Component 1 has N-terminal asp, glu, and his; these are probably residues 38, 11, and 105. Components 5, 6, and 7 have the same amount of N-terminal asp and lys, presumably corresponding to asp(NH_2) 34 or asp 38 and lys 1. Component 8 has N-terminal asp, ser, and lys, the amount of asp and ser being half that of lys; this is the result obtained by Ooi *et al.*, (1963) for component IV.

C-Terminal Group Analyses.—The C-terminal residues of the large components were determined with

TABLE V
C-TERMINAL AMINO ACIDS OF EACH COMPONENT RELEASED BY CARBOXYPEPTIDASE A AND B[a]

	1		5		6		7	8
	Native	Oxidized	Native	Oxidized	Native	Oxidized	Native	Native
Aspartic acid		0.25		0.12	0.46	0.30		0.30
Methionine sulfone		0.72		0.52		0.40		
Serine	0.83	0.93	0.22	0.69	2.0	1.09	0.66	1.13
Alanine	0.39	0.33	0.32	0.29	1.21		t[c]	0.59
Valine	0.56	1.00[b]	0.15	1.00[b]	0.8	1.00[b]	0.68	1.00[b]
Methionine	1.27		0.32		1.52		1.31	2.03
Tyrosine	0.53	0.68	t[c]		t[c]		t[c]	
Phenylalanine		0.26	t[c]	0.13	0.6	0.23	t[c]	
Lysine	2.0[b]	2.55	1.0[b]	1.10	1.18	0.57	1.00[b]	1.1
Histidine		0.21		0.10	0.48	0.26		0.32
Arginine	0.43		0.27		1.00[b]	0.71	0.38	0.58

[a] In moles of amino acid per mole of component. [b] This value was assumed and the other values in this column were computed relative to the assumed value. [c] t indicates trace.

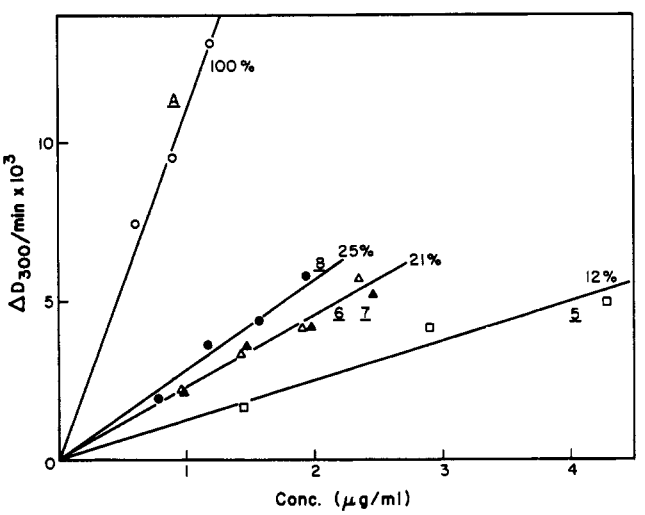

FIG. 8.—Dependence of initial rate of hydrolysis of RNA on the concentration of ribonuclease and its tryptic digestion products. The concentration of RNA was 1 mg/ml in 0.1 M, *p*H 5.0 sodium acetate buffer. The decrease in optical density at 300 mμ was measured at 25°. Ribonuclease A, O; component 8, ●; component 7, ▲; component 6, △; component 5, □.

carboxypeptidase A and B, and are listed in Table V. The analyses were carried out on both the "native" and oxidized forms of each component. Even though native ribonuclease is only partially digested by carboxypeptidase A (Sela *et al.*, 1957), the "native" components under consideration here do react because they undergo a thermal transition (Ooi and Scheraga, 1964) at the temperature (40°) at which carboxypeptidase digestion is carried out. Since these components were obtained by tryptic digestion, the C-terminal residues will be lysine or arginine, except for the C-terminal valine of ribonuclease. Therefore our interest lies in the other amino acids, besides these, which appear upon digestion with carboxypeptidase.

The results for component 1 suggest that the C-terminal residues are met 29–lys 31 and tyr 97–lys 98, as well as val 124. Components 5 and 7 have identical C-terminal residues, met 29–lys 31 and val 124. The presence of arginine in these components in small amounts compared to lysine indicates the presence of some contaminant. The results for component 6 suggest that the C-terminal residues are met 29–arg 33 and val 124. As with component IV (Ooi *et al.*, 1963), the C-terminal residues of component 8 appear to be met 29–lys 31, met 29–arg 33, and val 124. The differences in results between the native and oxidized forms of these components are attributable to incomplete release of some amino acids in the native form.

Summary of Results.—The N-terminal and C-terminal group analyses in Tables IV and V confirm the conclusions deduced from the amino acid composition data. Table III provides a summary of the bonds split and the amino acids missing in each component.

Stages of Digestion.—Now that the identities of the components of Figure 5 are known, it is possible to deduce the course of the digestion reaction at room temperature by following the appearance of the peaks. In the initial stages, cleavage occurs at lys 31–ser 32, arg 33–asp(NH_2) 34, or lys 37–asp 38, producing components 6 and 7. Since component 6 could not be isolated (Ooi *et al.*, 1963) in our previous work at 60° (whereas component 7, i.e., component III, could), we conclude that component 6 is less stable than component III to further tryptic attack at 60°. The next stage of digestion seems to be cleavage at the same bonds to produce component 5. Then component 1 is produced, i.e., an additional 16 amino acids are removed. Since no component, missing only the 16 amino acids, could be isolated, it is concluded that these 16 residues are removed after component 5 is formed. The time at which the lys 7–phe 8, arg 10–glu(NH_2) 11, lys 98–thr 99, and lys 104–his 105 bonds are split cannot be determined from these experiments.

Enzymic Activity.—Figure 8 shows the enzymic activity of each component toward RNA. Component 8, i.e., undigested component IV, has about 25% of the activity of ribonuclease A, components 6 and 7 have slightly less activity, and component 5 has 12% activity. It thus seems that the peptide chain from ser 32 to lys 37 is not essential for activity. However, these 6 amino acids must interact with the rest of molecule to stabilize its native conformation, since removal of the hexapeptide leads to a loss of up to 90% of the activity of the native molecule. This conclusion is supported by results of studies of the conformations of the various components isolated here (Ooi and Scheraga, 1964).

Effect of Split Bond on Disulfide Pairing.—C. B. Anfinsen (private communication) carried out an experiment in which the disulfide bonds of component IV were reduced and then reoxidized under conditions that have been shown to yield complete reactivation of the reduced intact chain of ribonuclease A (Epstein *et al.*, 1962). The reoxidized product from component IV showed no enzymic activity, in contrast to the correct disulfide pairing observed when all 123 peptide bonds of ribonuclease are intact. It may be that the

looser structure of component IV (Ooi and Scheraga, 1964), compared to that of ribonuclease A, contains a weaker driving force for the formation of the correct disulfide bonds.

Digestion with Chymotrypsin.—Experiments were carried out in which chymotrypsin rather than trypsin was added to components IV and III, respectively, at room temperature. It was found that digestion occurred *very slowly*; even after 24 hours the original material remained as the main peak of an IRC-50 chromatogram in both cases. Therefore it seems likely that components IV and III are similar in conformation to ribonuclease A, as far as susceptibility to chymotryptic attack is concerned. This result supports our earlier conclusion that the ribonuclease molecule is sufficiently folded near tyr 25 to prevent chymotryptic attack at room temperature (but permit it at 60°, after unfolding) (Rupley and Scheraga, 1963), and to prevent iodination of tyr 25 (Cha and Scheraga, 1963).

Discussion

One of the main results of this investigation is the observation that parts of the ribonuclease molecule between cys 26 and cys 40 can be cleaved without *complete* loss of activity. High temperature (~60°) is required for the first tryptic cleavage, to produce component IV (or 8). Subsequent cleavage can be produced at room temperature to form component III (or 7), which is missing the ser 32–arg 33 dipeptide, component 6, which is missing the asp(NH_2) 34–lys 37 tetrapeptide, and component 5, which is missing the ser 32–lys 37 hexapeptide. Apparently the side-chain groups of the hexapeptide are involved in holding the ribonuclease molecule in its native conformation. As successive cleavages occur in this region, the molecule loosens and loses activity. This conclusion is supported by physicochemical studies of these various digestion products, reported in the accompanying paper (Ooi and Scheraga, 1964).

Besides this main conclusion, other interesting aspects of the results may be cited. Only seven peptide bonds were cleaved by trypsin in the experiments carried out here, rather than the theoretically possible number of twelve. It seems that the remaining five peptide bonds are protected against tryptic attack by the compact folding of the digestion products. This conclusion, too, is supported by the physicochemical studies reported in the accompanying paper (Ooi and Scheraga, 1964). On the other hand, at high temperatures, these five peptide bonds would become exposed in the thermal unfolding and are digestible, as indicated by the different IRC-50 chromatograms which are obtained at high temperature (see Fig. 3).

Further, it may be noted that the lys 91–tyr 92 peptide bond was not cleaved in our experiments, even though it is in the tryptic digestion of S-protein (Allende and Richards, 1962). In our previous paper (Ooi *et al.*, 1963), we suggested that the N-terminal tail could protect the peptide bonds between cys 84 and cys 95 against attack by trypsin at high temperature. In the present work the lys 91–tyr 92 bond was not attacked by trypsin, even in component 1 which is missing part of the N-terminal tail from lys 1 to arg 10. Therefore we assume that the remaining part of the N-terminal tail, from glu(NH_2) 11 to ala 20, is the part which reacts with the chain between cys 84 and cys 95.

Another interesting fact is that no derivative could be isolated which contained the peptide from thr 99 to lys 104 but which was missing the N-terminal tail from lys 1 to lys 7. This suggests that the peptides from lys 1 to arg 10 and from thr 99 to lys 104 are closely related. That is, the N-terminal tail seems to cover the chain from around tyr 92 to lys 104. Since lys 7 and lys 41 are thought to be near each other, on the basis of experiments on dinitrophenylation (Hirs, 1962) and polyalanylation (Anfinsen, 1962) of ribonuclease A, we assume that the N-terminal tail protects the peptide cys 84–lys 104 from tryptic attack by taking a position such that lys 7 is in the region of lys 41. Furthermore, it seems necessary to remove the peptide from ser 32 to lys 37 in order to liberate the peptides from thr 99 to lys 104 and from lys 1 to arg 10, since no derivative was obtained which contained the peptide from ser 32 to lys 37, but was missing the peptides from lys 1 to arg 10 or from thr 99 to lys 104. The necessity to remove the peptide from ser 32 to lys 37 may explain why Allende and Richards (1962) could not obtain the component lacking the peptide from thr 99 to lys 104 in the tryptic digestion of S-protein. In order to satisfy all the requirements described above the N-terminal tail should be bent (providing an unfolded region) several residues before cys 26, say around the ala 20–ser 21 bond which can be split by subtilisin (Richards, 1955). These conclusions are supported by physicochemical investigations (Ooi and Scheraga, 1964) which indicate little change in conformation of derivatives IV, III, and 5 (i.e., 8, 7, and 5), and by the failure of chymotrypsin to attack components 7 and 8 at room temperature.

Recently Crestfield *et al.* (1963) suggested that his 12 and his 119 are near each other. From the results obtained here it appears that tyr 97, which is one of the buried tyrosines (Cha and Scheraga, 1963; Donovan, 1963) may also be near this pair of histidine residues.

Finally, the difficulty with which carboxypeptidase attacks the C-terminal tail of the "native" derivatives, compared to the oxidized ones, suggests that the C-terminus of the derivatives is partially buried, as it is in ribonuclease A, even at the transition temperature where the molecule is partially unfolded. This suggests that some folding remains at the transition temperature.

References

Allende, J. E., and Richards, F. M. (1962), *Biochemistry 1*, 295.

Anfinsen, C. B. (1962), *Brookhaven Symp. Biol. 15* (*BNL 738* (*C 34*)), 184.

Blackburn, S., and Lowther, A. G. (1951), *Biochem. J. 48*, 126.

Cha, C. Y., and Scheraga, H. A. (1963), *J. Biol. Chem. 238*, 2958, 2965.

Crestfield, A. M., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 2421.

Donovan, L. G. (1963), *Biochem. Biophys. Acta 78*, 474.

Epstein, C. J., Goldberger, R. F., Young, D. M., and Anfinsen, C. B. (1962), *Arch. Biochem. Biophys. Suppl. 1*, 223.

Hirs, C. H. W. (1962), *Brookhaven Symp. Biol. 15* (*BNL 738* (*C 34*)), 154.

Hirs, C. H. W., Moore, S., and Stein, W. H. (1960), *J. Biol. Chem. 235*, 633.

Ooi, T., Rupley, J. A., and Scheraga, H. A. (1963), *Biochemistry 2*, 432.

Ooi, T., and Scheraga, H. A. (1964), *Biochemistry 3*, 648. (this issue; accompanying paper).

Richards, F. M. (1955), *Compt. Rend. Trav. Lab. Carlsberg Ser. Chim. 29*, 329.

Rupley, J. A., and Scheraga, H. A. (1963), *Biochemistry 2*, 421.

Sela, M., Anfinsen, C. B., and Harrington, W. F. (1957), *Biochim. Biophys. Acta 26*, 502.

Smyth, D. G., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 227.