Structural Studies of Ribonuclease. VIII. Tryptic Hydrolysis of Ribonuclease A at Elevated Temperatures*

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Ribonuclease A was digested by trypsin at 60°, conditions under which the former protein undergoes a thermal transition. Chromatography and gel filtration of the digested material on IRC-50 and Sephadex columns yielded three major components, which were further purified on these same columns. It was found that one component was unreacted ribonuclease A, another was cleaved at lys-ser (31–32) and at arg-asp(NH_2) (33–34) [*i.e.*, the dipeptide ser-arg (32–33) was missing], and the other was a mixture of two components, one cleaved at lys-ser (31–32) and the other at arg-asp(NH_2) (33–34). These modified components all have the same enzymic activity toward ribonucleic acid (*viz.* 30%), and somewhat differing activity toward cytidine 2′–3′ cyclic phosphate. The results indicate that one of the parts of the ribonuclease molecule which unfolds in the thermal transition includes the region of the chain between lys 31 and asp(NH_2) 34, which also is not an essential part of the active center since activity remains in the modified components. The *N*-terminus of ribonuclease does not seem to unfold in the thermal transition.

In the previous paper (Rupley and Scheraga, 1963), experiments were reported on the action of chymotrypsin on ribonuclease at elevated temperatures, conditions under which the substrate undergoes a reversible thermal transition (Harrington and Schellman, 1956; Hermans and Scheraga, 1961). In this paper, we report the results of similar experiments in which trypsin was used as the proteolytic enzyme in the digestion of ribonuclease. Even though no significant amount of proteolysis occurred at room temperature, digestion did occur at elevated temperatures. Chemical analysis of the digestion products permitted identification of the specific peptide bonds which were hydrolyzed and, therefore, of a region of the molecule undergoing the reversible thermal transition.

It is also of considerable interest that three purified high-molecular-weight components of the digestion mixture exhibited enzymatic activity toward both ribonucleic acid (RNA) and the synthetic substrate cytidine 2′-3′ cyclic phosphate.

Experimental

Materials.—Essentially all of the materials used have been described in the previous paper (Rupley and Scheraga, 1963). In addition, two-times crystallized trypsin and diisopropylfluorophosphate-treated carboxypeptidase B were obtained from Worthington. Magnesium sulfate in the trypsin preparation was replaced by 0.001 N HCl by gel filtration on a Sephadex G-25 column.

Tosyl arginine methyl ester (TAMe) was obtained from Mann Biochemical Company. It was used for assays of trypsin activity.

Cytidine 2′-3′ cyclic phosphate was obtained from Schwarz Biochemicals.

Method.—Digestion of ribonuclease A with trypsin was carried out in 0.01 M KCl, *p*H 6.5, 60°, in a *p*H stat (Radiometer TTT1) as described in the previous paper (Rupley and Scheraga, 1963). A concentration of ribonuclease A of 10 mg/ml was used routinely. Trypsin loses its activity rapidly at high temperature in a TAMe hydrolysis experiment; however, trypsin is slightly more stable than chymotrypsin at elevated temperatures. Therefore, it was necessary to add successive aliquots of trypsin to the solution in order to obtain a sufficient degree of digestion of ribonuclease. The amount of base consumed during the digestion was recorded with the *p*H stat. Also, aliquots of the solution were removed at various stages of the digestion, and enzymic activity and increase in ninhydrin color were measured.

In the preparative experiments, diisopropylfluorophosphate was added to about 10 times excess of trypsin in order to stop the reaction. The solution was then cooled quickly to 0° and lyophilized.

IRC-50 chromatographic analyses of digestion mixtures were performed using columns of Amberlite IRC-50, XE-64 resin, in conjunction with a Technicon Autoanalyzer, as described in the previous paper.

Gel filtration on Sephadex G-75 and G-25 was used to remove salts and small peptides from the large components of the chromatographically separated digestion mixture. Ammonium acetate (0.05 M) was the supporting electrolyte in the gel filtration, and the volatile salt was removed from the isolated fractions by repeated lyophilization. For further purification, the same procedure using the IRC-50 and Sephadex columns was repeated (usually twice) until chromatographically pure components were obtained.

All column chromatography (IRC-50 and Sephadex) was carried out at room temperature.

Performic acid oxidation was carried out according to Hirs (1956) at −10°.

C-Terminal group analyses were performed with diisopropylfluorophosphate-treated carboxypeptidase B mixed with carboxypeptidase A under the same conditions as described in the previous paper. The concentration of carboxypeptidase B was one fiftieth that of carboxypeptidase A. Digestion was carried out at 40° for 6–8 hours in 0.5 M phosphate buffer at *p*H 8.0.

N-Terminal group analyses were performed by the

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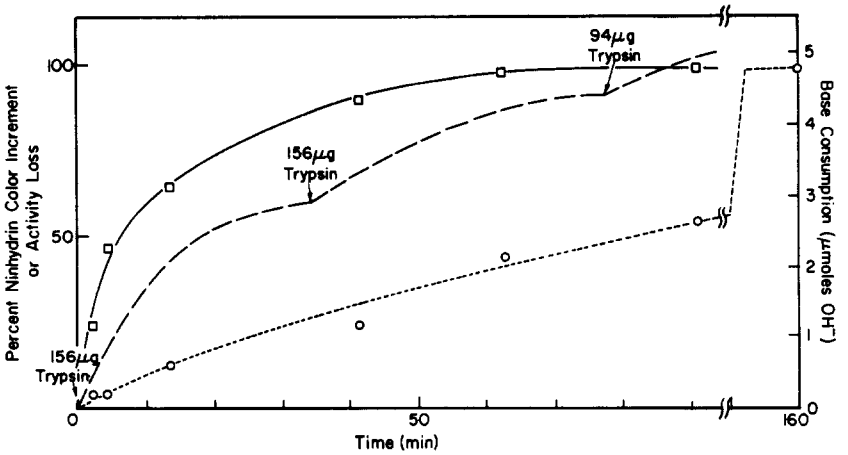

FIG. 1.—Time dependence of digestion of ribonuclease A by trypsin. Initial concentration of ribonuclease A: 300 mg/30 ml. Arrows indicate the addition of trypsin. Digestion conditions: 0.01 M KCl, *p*H 6.5, 60°. The large-dashed line represents base consumption, the circles increase in ninhydrin color, and the squares loss of activity.

fluorodinitrobenzene method, as described in the previous paper.

Amino acid analysis, analytical scale chromatography on IRC-50 columns, and analysis of the *C*-terminal amino acids released by the mixture of carboxypeptidases were carried out with the Technicon Amino Acid Analyzer, as described in the previous paper.

Ribonuclease activity toward ribonucleic acid was measured by the spectrophotometric method of Kunitz (1946) with RNA at 1 mg/ml in 0.1 M sodium acetate buffer at *p*H 5.0, 26°. A solution of 0.11 M $NaNO_3$ was used as the reference solution. The activity toward cytidine 2′-3′ cyclic phosphate was also determined spectrophotometrically by the measurement of an increase in optical density at 284 mμ (Crook *et al.*, 1960). Several substrate concentrations (near 0.1 mg/ml in 0.2 M Tris buffer, *p*H 7.2) were used. The measurements were carried out at 25°.

RESULTS

Digestion.—The time dependence of the rate of tryptic hydrolysis of ribonuclease A at 60° is shown in Figure 1, where the course of the digestion was measured by base uptake, per cent increase in ninhydrin color, and loss of activity. The times at which successive aliquots of trypsin were added to compensate for the thermal inactivation of trypsin are indicated. The initial high rate of base consumption was accompanied by a corresponding loss of ribonuclease activity; the subsequent decrease in initial rate was presumably due to the denaturation of trypsin, as well as to a decrease in the concentration of unhydrolyzed ribonuclease. Complete digestion was achieved by adding trypsin several times until no further base uptake occurred; a total of 1.9 mg of trypsin was added to achieve complete digestion. The digestion mixture became turbid after about 3 μmoles of base were consumed. A similar observation was made in the case of chymotryptic digestion.

In order to express the data for the extent of digestion, the results are summarized by plotting the per cent increase in ninhydrin color or loss of activity against the base uptake, as shown in Figure 2. In this figure, two experiments are shown, one using 300 mg ribonuclease A and the other 800 mg ribonuclease A. The changes in ninhydrin color and activity lie on the same curves in both experiments. This result indicates that the increase in ninhydrin color or activity loss is related to the base consumption, independent of the amount of ribonuclease digested. Therefore, it was convenient to follow the digestion in subsequent preparative experiments by measuring only the base consumption.

However, it is difficult to relate base consumption to the number of peptide bonds hydrolyzed, since the *pK* values of the new α-amino groups in the digested material are unknown. Therefore, the extent of hydrolysis was calculated from the per cent increase in ninhydrin color, with 8.5% increase being *assumed* to correspond to one bond split. On this basis, the loss of activity may be plotted against the average number of bonds split (Fig. 3). The data indicate that about half the enzymic activity was lost after the splitting of an average of one or two bonds. This observation is in agreement with the rapid appearance (discussed below) of several products of low enzymic activity. Ultimately 11 to 12 bonds were hydrolyzed; in this connection, the theoretical number of bonds in ribonuclease which can be cleaved by trypsin is 12. Therefore, the splitting of one or two bonds in the partially unfolded molecule probably leads to further changes in conformation which enable the digestion to proceed to completion at high temperature.

Chromatographic Separation of the Products of Tryptic Digestion of Ribonuclease.—The digestion products

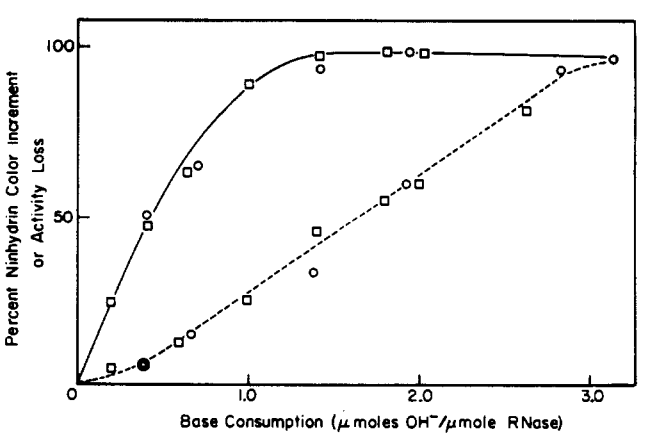

FIG. 2.—The increase in ninhydrin color, and loss of activity, shown as a function of base comsumption. Initial amounts of ribonuclease A were: □, 300 mg/30 ml; ○, 800 mg/80 ml. The solid line represents the loss of activity and the dashed line the increase in the ninhydrin color.

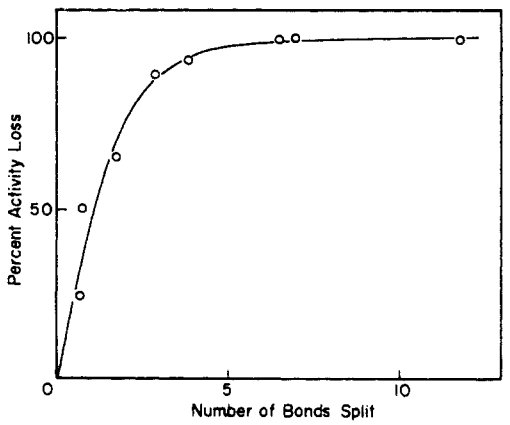

FIG. 3.—Loss of activity as a function of the number of bonds cleaved, computed from the increase in ninhydrin color.

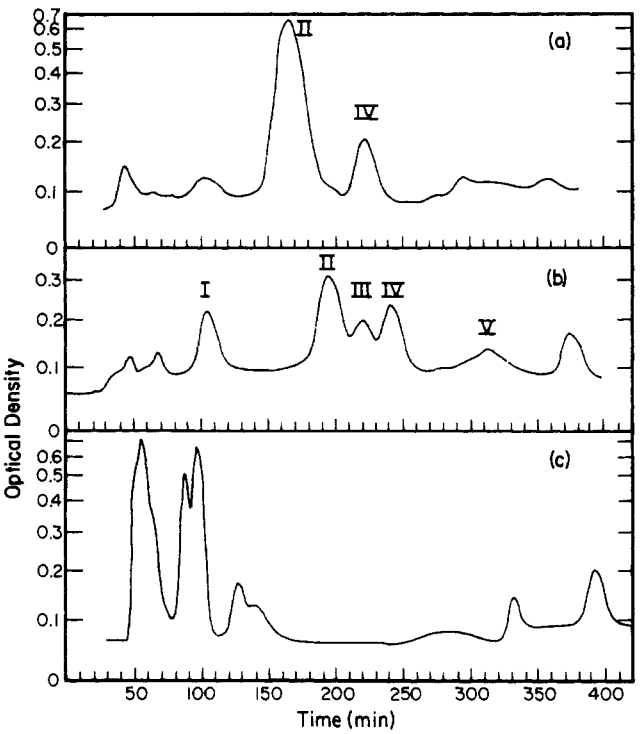

FIG. 4.—Chromatographic analyses of tryptic digests of ribonuclease after three stages of digestion at 60°. (*a*) Short-time, (*b*) medium-time, and (*c*) exhaustive digestion. The ordinate represents the optical density of the effluent after reaction with ninhydrin, and the abscissa is the effluent volume expressed on a time scale on which 0.32 ml corresponds to one minute.

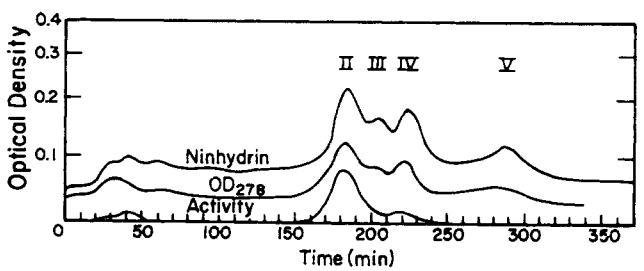

FIG. 5.—Chromatographic analysis on IRC-50 of a medium-time tryptic digest which had first been separated on a Sephadex column. The coordinates are the same as in Fig. 4. Enzymic activity is expressed as an optical density at 278 mμ.

were analyzed on a 1 × 30 cm column of IRC-50 resin. The results of three runs, representing a short-time digestion (*ca.* 1 bond split), a medium-time digestion (*ca.* 3 bonds split), and an exhaustive digestion (*ca.* 11 to 12 bonds split), are shown in Figure 4. In the earliest stage of the digestion, four major peaks appeared in the chromatogram (Fig. 4*a*); the large main component was probably unreacted ribonuclease A, and the other three were new components. In the second stage, several new peaks appeared (Fig. 4*b*); the three peaks which are eluted between 200 and 250 minutes are of special interest in this pattern (see below). In Figure 4*c*, it is seen that no components remained in the middle part of the chromatogram after exhaustive digestion.

In order to estimate the relative sizes of the components in the digestion mixtures, the products of a medium-time digestion were first separated on a column of Sephadex G-75. The only materials collected were those which eluted with the front; these were presumably components with a molecular weight of the order of that of ribonuclease. This eluate, after desalting by lyophilization, was then analyzed on an IRC-50 column under the same conditions used above. In this experiment, one third of the sample was developed for ninhydrin color and analyzed on the autoanalyzer, and two thirds was collected for the measurement of optical density at 278 mμ and activity against RNA. The resulting patterns are shown in Figure 5. From Figure 5 it can be seen that three major peaks were eluted in the middle of the chromatogram; these are designated components II, III, and IV. A minor component (V) was eluted after component IV. Components II–V are thus of large molecular weight (of the order of that of ribonuclease). The other peaks (including component I) in the original IRC-50 chromatogram (Fig. 4*b*) may be regarded as low-molecular-weight peptides. From the position on the chromatogram, the peak height, and the activity, we may assume that peak II corresponds to unreacted ribonuclease A; components III, IV, and V are then modifications produced by tryptic digestion.

Our primary interest lies in the large-molecular-weight components produced in the early stages of the reaction. Figure 6 presents the chromatographic patterns of ribonuclease A after various stages of digestion (and after separation of the larger components on the Sephadex column as was done in the above experiment). It can be seen that peak IV appeared in the early stages of the reaction; peak III, in between peaks II and IV, appeared at a later stage in the reaction. Comparison of the relative heights and appearance of the peaks seems to confirm that peak IV is the first product to appear and that peak III is the next one. As in the case of the intermediates in chymotryptic digestion, these components moved somewhat more slowly on the column with increasing degree of digestion.

Identification of Bonds Cleaved in High-Molecular-Weight Intermediates.—The components of a preparative-scale medium-time digestion mixture were separated, first on IRC-50 and then on Sephadex, under the same conditions as described above, to obtain pure components. Figure 7 shows the IRC-50 chromatograms of each isolated and purified component to indicate their homogeneity. Fractions II, III, and IV each contained less than 10% chromatographically detectable impurities. The yields from the medium digest were 20%, 4%, and 6% for purified components II, III, and IV, respectively, based on the original quantity of ribonuclease A used.

The amino acid compositions of each component are listed in Table I. The results show that components

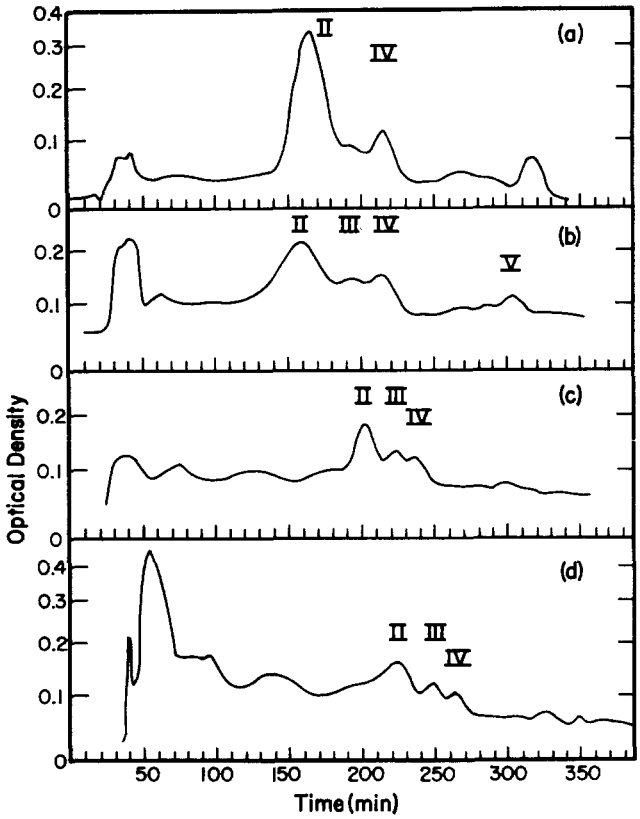

FIG. 6.—Chromatographic analyses on IRC-50 of large components of several tryptic digests (obtained by preliminary separation on a Sephadex column). The time of digestion increases from *a* to *d*; the patterns thus show the course of the appearance of new components. The extent of digestion, in terms of the number of bonds split, was: (*a*) 1 or 2; (*b*) 2 or 3; (*c*) 3 or 4; (*d*) 4 or 5.

II and IV had the same composition as ribonuclease A, indicating that no amino acids are missing as a result of the tryptic hydrolysis. However, component III lacked one mole of serine and one mole of arginine, the same deletions found by Allende and Richards (1962) in the tryptic digestion of *S*-protein.

N-Terminal group analyses of these components were carried out by the fluorodinitrobenzene method, and

TABLE I
AMINO ACID COMPOSITION
(Moles of amino acid residue per mole of component)

	Ribonuclease A				
	Theor.	Exp.	II	III	IV
Aspartic acid	15	15.6	15.4	15.6	15.0
Threonine[a]	10	9.8	9.75	9.9	9.8
Serine[a]	15	15.2	14.7	14.2	15.0
Glutamic acid	12	12.7	12.5	12.7	12.7
Proline	4	4.15	3.7	3.89	3.85
Glycine	3	2.89	2.94	3.29	3.0
Alanine	12	11.5	11.7	12.0	11.6
Half-cystine	8	8.3	8.2	8.5	8.2
Valine	9	8.6	8.9	8.85	8.7
Methionine	4	3.6	3.95	3.9	3.89
Isoleucine	3	2.16	2.22	2.28	2.22
Leucine	2	1.97	1.97	1.97	2.06
Tyrosine[a]	6	6.35	6.25	6.35	6.1
Phenylalanine	3	2.81	2.80	2.92	2.83
Lysine	10	9.7	9.7	9.8	10.2
Histidine	4	3.94	3.9	3.72	3.95
Arginine	4	3.75	4.05	3.17	3.95

[a] These data include the following correction factors for hydrolysis losses: thr (1.05), ser (1.12), tyr (1.14).

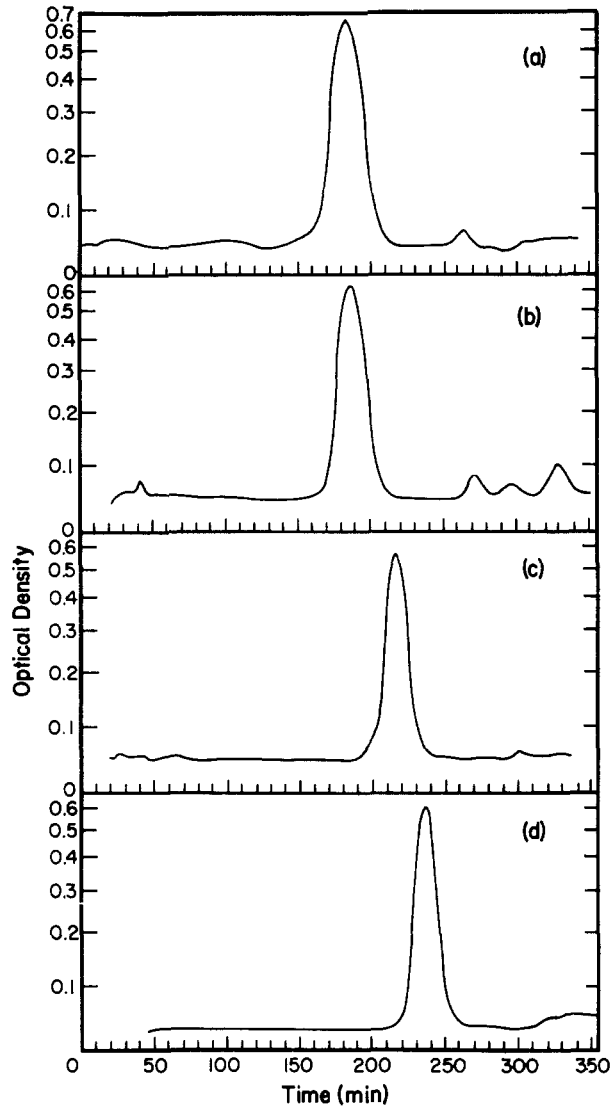

FIG. 7.—Chromatographic analyses on IRC-50 of ribonuclease A and purified components II, III, and IV, shown in this order from (*a*) to (*d*).

the results are shown in Figure 8. The recoveries of the DNP-amino acids were usually *ca.* 70%. In representing the data in Figure 8, the values for di-DNP-lysine have been normalized to 100% and the other values corrected accordingly. From Figure 8, it can be seen that component II contained mainly *N*-terminal lysine; this is additional evidence that component II is unreacted ribonuclease A. Component III contained equal amounts of di-DNP-lys and DNP-asp, while component IV had equal amounts of DNP-asp and DNP-ser together with twice as much di-DNP-lys. The small amount of DNP-asp in component II was probably due to some contamination from component III.

C-Terminal group analysis of these components was carried out on performic acid–oxidized materials with a mixture of carboxypeptidases A and B. In computing the number of residues liberated by carboxypeptidase, it was assumed that the *C*-terminal residue, valine, of oxidized ribonuclease A was quantitatively released; the data for the other amino acids (shown in Table II) were then normalized to this value. It can be seen that the *C*-terminal residues up to histidine 119 were liberated in both ribonuclease A and component II—further evidence that component II is unreacted ribonuclease A. Components III and IV contained

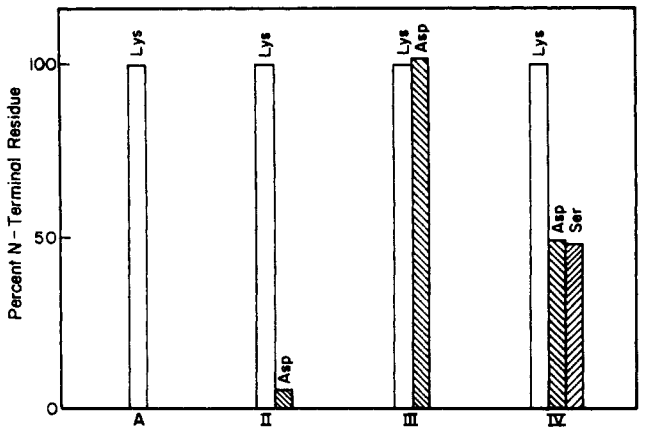

FIG. 8.—*N*-Terminal residues of ribonuclease A and components II, III, and IV by the fluorodinitrobenzene method. The data were computed by assuming that the recovery of *N*-terminal lysine is 100% (the actual recovery of lysine was 70%).

the same *C*-terminal residues as ribonuclease A; however, in addition to these residues, component III exhibited lys and met, and component IV exhibited lys, met, arg, and ser. In the determination of the amino acids released by carboxypeptidase, several small peptides moved on the column, giving rather broad peaks in contrast to those given by amino acids. Fortunately, these peptide peaks did not coincide with the main amino acids peaks of the *C*-terminal residues.

TABLE II
CARBOXYPEPTIDASE A AND B DIGESTION PRODUCTS
(Moles of amino acid residue per mole of component from oxidized species)

	A	II	III	IV
Aspartic acid	0.59	0.62	0.58	0.59
Methionine sulfone	t	t	0.84	0.72
Threonine	p	p	p	p
Serine	0.97	0.97	0.96	1.14
Glutamic acid	t	t	t	t
Proline				
Glycine	t	t	t	t
Alanine	0.76	0.75	0.83	0.79
Cystine				
Valine	1.00	1.00	1.00	1.00
Methionine				
Isoleucine	p	p	p	p
Leucine	p	p	p	p
Tyrosine	p	p	p	p
Phenylalanine	0.70	0.71	0.54	0.65
Lysine		t	0.92	0.76
Histidine	0.76	0.70	0.67	0.63
Arginine		t	t	0.42

t, trace; p, peptide (broad peak of small area appeared in the chromatogram in the positions where these amino acids normally appear).

On the basis of the data of Tables I and II and Figure 8, it is possible to identify the chemical structure of each major component of the digestion mixture. Component II is clearly unreacted ribonuclease, since both have the same amino acid composition and *N*- and *C*-terminal amino acids; therefore, no peptide bonds have been split in component II. Component III is a molecule which is missing the ser-arg (32–33) dipeptide, since its amino acid composition indicates that one mole of serine and one mole of arginine are missing. In support of this conclusion, the *N*-terminal residues of component III are lysine (the *N*-terminal residue of ribonuclease A) and aspartic acid (or asparagine, presumably asparagine 34), and its *C*-terminal residues are that of ribonuclease A and also a lysine (presumably residue 31) followed by methionine (residues 30 and 29). Component IV is a mixture of two molecular species, one with the lys-ser (31–32) bond cleaved and the other with the arg-asp (NH_2) (33–34) bond cleaved. This identification is based on the identity of the amino acid compositions of component IV and ribonuclease A (indicating no missing amino acids), on the *N*-terminal residues (equal amounts of ser-32 and asp-(NH_2)-34 and twice as much lys-1), and on the *C*-terminal residues (half a mole of arg-33, followed by ser, lys, and met, but the amount of lys-31 is more than half a mole, indicating that lys-31 is also *C*-terminal). Since no other positions in the sequence of ribonuclease A (Hirs *et al.*, 1960) can meet these requirements, it is safe to conclude that the two species present in component IV each have one bond split, *viz.*, lys-ser (31–32) and arg-asp(NH_2) (33–34), respectively.

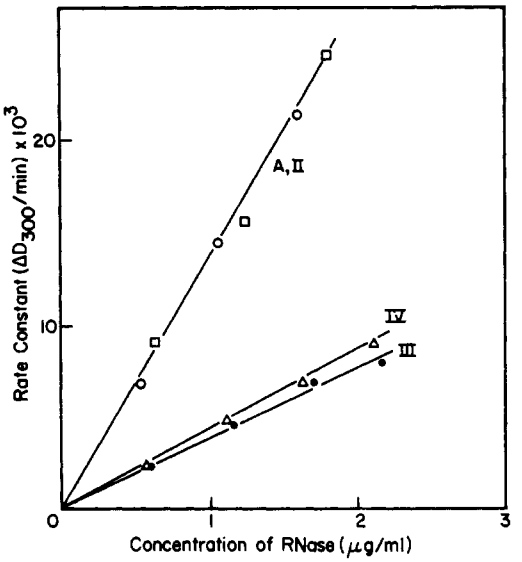

FIG. 9.—Concentration dependence of the initial rate constant for the hydrolysis of RNA by components II, III, and IV and ribonuclease A. ○, ribonuclease A; □, component II; ●, component III; △, component IV.

It has already been mentioned that component IV appeared earlier in the digestion than did component III. A similar sequence of analyses was carried out on a sample of component IV isolated from an early-time digest before an appreciable amount of component III had appeared; the same conclusions about the chemical structure of component IV were reached. Therefore, we may conclude that cleavage can occur with equal probability at lys-ser (31–32) in some molecules and at arg-asp(NH_2) (33–34) in others, and that the second bond split is one or the other of these two bonds to liberate the dipeptide ser-arg (32–33) which is missing in component III.

These results indicate that one of the regions of the polypeptide chain in which the thermal transition occurs is in the vicinity of residues 31 to 34.

Activity of the Modified Components.—Preliminary activity measurements, with RNA as substrate, showed that components III and IV had some enzymic activity. In order to confirm the existence of this activity, the dependence of the rate constant for depolymerization of RNA on enzyme concentration was determined. The results, shown in Figure 9, indicated that component II had the same activity as ribonuclease A, confirming that this component is unreacted ribonuclease A. Component IV exhibited 30% of the activity

of component II and ribonculease A, and component III had slightly less activity than component IV.

Measurements of the activity of ribonuclease A and the several modifications toward cytidine 2'-3' cyclic phosphate were analyzed by considering the inverse of the velocity of hydrolysis ($1/V$) as a function of the inverse of the substrate concentration ($1/S$). The experimental plots were linear, and the values of V_m and K_m obtained for each component are listed in Table III.

TABLE III
KINETIC CONSTANTS OF COMPONENTS

	Ribonuclease A	IV	III
K_m (mg/ml)	0.75	1.4	1.8
V_m (relative to ribonuclease A)	1.0	0.25	0.14

The results indicate that the splitting of one bond (to produce component IV) gives rise to a greater change in K_m and V_m than does the splitting of the second bond (to produce component III), suggesting that most of the conformational change (presumed to be responsible for the loss in activity) arises in the conversion of ribonuclease A to component IV rather than in the conversion of component IV to component III.

DISCUSSION

The *N*-terminal tail from lys-1 to tyr-25 has two positions which are cleaved by trypsin in oxidized ribonuclease (Hirs *et al.*, 1956), *viz.*, lys-phe (7–8) and arg-glu (10–11). However, since these bonds are not split in the early stages of the digestion of ribonuclease A at 60°, the *N*-terminal tail near these bonds probably is not unfolded in the thermal transition. Also, it is known that the *N*-terminus is required for enzymic activity (Richards, 1955). Therefore, the finding that components III and IV have appreciable enzymic activity is consistent with the observation that no bonds of the *N*-terminal tail were split. Since subtilisin can split the ala-ser (20–21) bond at room temperature (Richards, 1955), and trypsin can split the *S*-peptide at room temperature (Allende and Richards, 1962), the first few residues of the *N*-terminus of native ribonuclease are probably protected. The results of the previous paper (Rupley and Scheraga, 1963) indicate that unfolding occurs near tyr-cys (25–26), leading to chymotryptic cleavage of this bond.[1] Therefore, presumably the region between residues 25–35, in which two primary tryptic hydrolyses are located, unfolds in the thermal transition without disruption of at least the first 10 or 12 residues of the *N*-terminus of the molecule.

It is interesting to note that the peptide bond lys-tyr (91–92) in the *S*-protein was attacked by trypsin at room temperature (Allende and Richards, 1962), but was not attacked in the partially unfolded molecule at 60°. Moreover, the dipeptide ser-arg (32–33) was missing from the *S*-protein after tryptic digestion. Therefore, some of the peptide bonds between half-cystines VI and VII are probably protected by the *N*-terminal tail. This conclusion is not inconsistent with the observed chymotryptic split at tyr-lys (97–98) (Rupley and Scheraga, 1963); presumably the protection afforded by the *N*-terminal tail to the 91–92 bond does not extend to the 97–98 bond.

Since components III and IV have about 30% activity toward RNA, some conformational change must occur as a result of the splitting of even one peptide bond in the region between half-cystines I and II. However, since activity is not completely lost by the splitting of this bond, the groups required for enzymic activity must still occupy approximately the same spatial arrangements in components III and IV as they do in ribonuclease A.

Physicochemical measurements on components III and IV are now in progress.

[1] Since the *N*-terminal tail is removed by chymotrypsin at 60° it is not surprising that this digestion product has no enzymic activity.

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