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Structural Studies of Ribonuclease. VII. Chymotryptic Hydrolysis of Ribonuclease A at Elevated Temperatures*

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Ribonuclease A undergoes a transition at elevated temperatures which renders certain peptide bonds accessible to chymotryptic hydrolysis, leading to the production of large intermediates in the proteolytic reaction. These intermediates have been isolated and the sites of chymotryptic attack have been identified as tyr-cys (25–26), met-ser (79–80), tyr-lys (97–98), leu-thr (35–36), tyr-ser (76–77), phe-val (46–47), and an unidentified bond whose *N*-terminal member is glutamic acid or glutamine. The data suggest that in the thermal transition three regions of the ribonuclease polypeptide chain unfold, around residues 73 to 80, around half-cystine 26, and around tyrosine 97. The *C*-terminal region of the molecule, including tyrosine 115, probably is not affected by the thermal unfolding. Several other structural implications of the data are discussed, and the effects of solvent and temperature changes on the proteolytic reaction are considered.

Recent work in this laboratory and elsewhere (Harrington and Schellman, 1956; Hermans and Scheraga, 1961a,b; Tanford and Weber, 1959 [private communication]; Holcomb and Van Holde, 1962) on the reversible thermal transition of ribonuclease indicates that the native molecule is only partially unfolded in the transition. This paper presents experiments locating the portions of the polypeptide chain which unfold; these experiments are based on the hypothesis that the unfolded parts of a polypeptide chain are more susceptible than folded regions to attack by proteolytic enzymes (Mihalyi and Harrington, 1959). The results of experiments utilizing chymotrypsin as the proteolytic enzyme are described below; the use of trypsin will be discussed in the accompanying paper (Ooi *et al.*, 1963).

In order to relate the sites of chymotryptic hydrolysis to the unfolding of the molecule, several sets of facts must be established. First, the proteolysis must be clearly associated with the unfolding reaction; in this connection, both chymotrypsin and trypsin fail to hydrolyze native ribonuclease A at an appreciable rate (Spackman *et al.*, 1960; Scheraga and Rupley, 1962) but, as shown below and in the following paper (Ooi *et al.*, 1963), these enzymes rapidly hydrolyze the thermally unfolded molecule. Second, large intermediates in the proteolysis, in which only one or a few bonds have been hydrolyzed, must be isolated; if proteolysis is too extensive it is not possible to distinguish bonds broken as a result of their exposure in the transition from bonds broken at random (except for the requirements of enzymic specificity). Finally, the position of the splits must be determined by chemical analysis of the large intermediates isolated, a procedure greatly facilitated by the recent description of the amino acid sequence of ribonuclease A (Hirs *et al.*, 1960; Spackman *et al.*, 1960; Smyth *et al.*, 1962; Potts *et al.*, 1962; Gross and Witkop, 1962; Smyth *et al.*, 1963). A discussion of previous work on the limited proteolysis of ribonuclease may be found in a review by Scheraga and Rupley (1962).

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EXPERIMENTAL

Materials.—Ribonuclease was purchased as the crystalline material from Sigma and from Wilson Chemical Companies. The ribonuclease A fraction was obtained by chromatography of the crystalline protein on unsieved Amberlite IRC-50, XE-64 in 0.2 M sodium phosphate buffer, *p*H 6.47 (Hirs *et al.*, 1953), 7.5 × 60 cm column, and was deionized by passage through the mixed-bed ion exchanger, MB-1 (Rohm and Haas Co.) 5.0 × 40 cm column.

Ribonucleic acid (RNA) was purchased from Nutritional Biochemicals Co. and was used without further purification.

Diisopropylfluorophosphate was obtained as a gift from B. J. Jandorf of the Army Chemical Center, Maryland, and was used as a 0.1 M solution in dry isopropanol.

Acetyltyrosine ethyl ester and carbobenzoxyglycylphenylalanine were products of Mann Research Laboratories.

Sephadex (G-25 and G-75) was obtained from Pharmacia.

Diethylaminoethyl cellulose (DEAE-cellulose) was obtained from the Brown Company.

Fluorodinitrobenzene and trimethylamine were Eastman Kodak White Label products.

Tris buffer was obtained from Sigma Chemical Company.

Chymotrypsin was purchased as the crystalline material from Sigma Chemical Company, and carboxypeptidase A (diisopropylfluorophosphate-treated) and leucine aminopeptidase from Worthington.

Ninhydrin and hydrindantin were obtained from the Dougherty Chemical Company.

Other chemicals were reagent grade where possible.

Methods.—Digestion of ribonuclease A with chymotrypsin was performed at constant *p*H with the Radiometer *p*H-stat (units TTT-1, SBR-2, SBU-1). The progress of the reaction was followed by measurement of the change in ninhydrin color (Moore and Stein, 1954), enzymic activity, and the volume of base required to maintain a constant *p*H.

Reaction mixtures routinely contained about 10 mg/ml ribonuclease A, 0.01 M KCl or $CaCl_2$, 0.05 to 0.5 mg/ml chymotrypsin, and other reagents as required; the *p*H was maintained at 6.5, the temperature at 25° to 60° ± 0.5°. A difficulty in the reactions at higher temperatures was the rapid loss of chymotryptic activity at 60°; for example, in the absence of substrate, at *p*H 6.5, 0.01 M $CaCl_2$, and 60°, less than 5 minutes were required for 50% inactivation. In the absence of calcium ions the loss of activity was more rapid. Although the presence of substrate (*i.e.*, ribonuclease A) reduced the rate of inactivation, several successive additions of chymotrypsin were required to obtain moderate or extensive chymotryptic hydrolysis of ribonuclease A at 60°, leading to final concentrations of chymotrypsin of approximately 0.5 mg/ml. Probably associated with this inactivation of the chymotrypsin was a turbidity which appeared shortly after addition of the chymotrypsin at 60° and increased with time. The reaction was terminated by the addition of diisopropylfluorophosphate to a concentration of 0.01 M, followed by cooling to 0°. The digestion mixture was then lyophilized if concentration of the products was necessary.

Ribonuclease activity was measured by the change in spectrum (Kunitz, 1946) which occurs in the enzymic depolymerization of RNA in 0.1 M sodium acetate buffer at *p*H 5.01. Chymotryptic activity was measured titrimetrically by the amount of base required to maintain a constant *p*H in the enzymic hydrolysis of acetyl tryosine ethyl ester (Schwert *et al.*, 1948; Cunningham, 1954).

IRC-50 chromatographic analyses of digestion mixtures were performed on columns of Amberlite IRC-50, XE-64, prepared according to Hirs *et al.* (1953). The column effluent was monitored with use of the Technicon Autoanalyzer to develop and record the ninhydrin color; a gradient in the influent buffer was produced with the Technicon Autograd (modeled after the Varigrad of Peterson and Sober, 1959), in which the first three chambers contained 35 ml each of 0.15 M sodium phosphate, 0.001 M sodium versenate buffer, *p*H 6.40, and the fourth chamber contained 35 ml of 1.0 M sodium phosphate, 0.001 M sodium versenate buffer, *p*H 6.47. The gradient thus exhibited a sharp rise in the last third of the elution to 1.0 M sodium phosphate at the end of the analysis. A flow rate of about 23 ml/hour resulted in adequate resolution and a total analysis time of 6 hours. The column load could be varied from 3 to 30 mg with good reproducibility and resolution of digestion mixtures. When desired, the effluent stream was split and fractions collected for measurement of enzymic activity and absorbancy at 278 mμ. A steeper or a shallower gradient (obtained by use of three or five chambers of the Autograd, respectively) led to poorer resolution of the chymotryptic digest components. Doubling the volume of the gradient while maintaining the same shape of gradient curve led to improved resolution and was used in preparative chromatography of the digests but was not routinely employed because of the greater time of analysis required.

Gel filtration on 2.8 × 30 cm columns of Sephadex G-75 was used to desalt and to isolate heavy components from digestion mixtures. The supporting electrolyte was 0.05 M ammonium acetate, and the volatile salt was removed from the isolated fractions by lyophilization (repeated three or four times). In the absence of salt, ribonuclease A moved slowly on Sephadex, in agreement with the observations of other workers (Glazer and Wellner, 1962). Desalting of IRC-50 fractions was accomplished by gel filtration on Sephadex G-25, employing columns 2.8 × 60 cm with 0.05 M ammonium acetate as supporting electrolyte, followed by repeated lyophilization.

DEAE-cellulose chromatographic analyses were performed on 1 × 30 cm columns of DEAE-cellulose in sodium borate buffer, *p*H 9.5. The column effluent was monitored with the Technicon Auto Analyzer, and the influent gradient was produced with the first and second chambers of the Autograd, which contained, respectively, 200 ml 0.005 M sodium borate buffer, *p*H 9.5, and 190 ml 0.1 M sodium chloride, 0.025 M sodium borate buffer, *p*H 9.5.

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

Hydrolysis of protein samples was performed by heating in 6 N HCl at 110° for 22 hours in evacuated and sealed ampuls (Gundlach *et al.*, 1959).

Amino acid analyses were performed with the Technicon Amino Acid Analyzer. The data were interpreted by assuming for glutamic acid and alanine an appropriate number of residues per molecule, and then calculating for the other amino acids the number of residues per molecule relative to the two assumed (see Gundlach *et al.*, 1959). This procedure was necessary since only small quantities of material were available, and consequently the concentration of the sample to be analyzed could not be accurately determined. Relative areas of the peaks in analyses of the same standard sample of amino acids (purchased from the California Corporation for Biochemical Research) routinely were reproducible to within 3%. Corrections were applied for hydrolysis losses for serine (89% recovery assumed), threonine (95%), tyrosine (88%), cystine (81%), and cysteic acid (84%); the first four values were determined by Gundlach *et al.* (1959), and the last was determined in this laboratory.

Fluorodinitrobenzene was used to determine *N*-terminal groups. The reaction was carried out according to Hirs (1956), with trimethylamine as buffer. After lyophilization the protein was hydrolyzed by heating 18–20 hours at 110° in evacuated and sealed ampuls. Paper chromatography of the ether-soluble derivatives was performed in one dimension with the use of Levy's (Fraenkel-Conrat *et al.*, 1955) toluene solvent; analysis in one dimension was adequate to identify all the DNP-amino acids obtained (after a suitable series of comparisons with standards had been made) except for glutamic acid, which was eluted and rerun in phosphate buffer (Fraenkel-Conrat *et al.*, 1955). Paper electrophoresis of the aqueous fraction

was performed according to Anfinsen (private communication); 10% glacial acetic acid and 500 volts were used for 4 hours. Corrections for hydrolysis and chromatographic losses were determined by carrying two samples of synthetic mixtures through the analytical procedure; the recoveries were 45% for glutamic acid, 62% for serine, 64% for threonine, 78% for valine, 58% for lysine, 4% for cystine, 13% for cysteic acid. The nearly complete destruction of cystine and cysteic acid prevented their detection in the analysis. The high losses observed for the other relatively stable derivatives may have been a result of the small amounts analyzed (about 0.3 μmole, equivalent to 5 mg of ribonuclease).

Leucineaminopeptidase and *carboxypeptidase A, diisoprovylfluorophosphate treated*, were used for the analysis of *N*- and *C*-terminal residues, respectively. The reactions were performed under conditions similar to those described by Hirs *et al.* (1960), and the amino acids released were determined by column analysis.

*p*H was measured by Beckman Model-G and Radiometer TTT-1 instruments. Buffer solutions of standard *p*H (*p*H 4 and 7) were obtained from Beckman or were prepared (phosphate buffer, *p*H 6.86) according to Bates (1954).

Results

Rate of Hydrolysis of Ribonuclease by Chymotrypsin, Its Correlation with the Thermal Transition, and Its Alteration Due to Changes in Solvent Composition.—Initial experiments were carried out to determine the rate of hydrolysis of ribonuclease by chymotrypsin at various temperatures. In a typical experiment, at 40.3°, a solution of ribonuclease A, 8.95 mg/ml, in 0.01 M $CaCl_2$ was equilibrated under nitrogen in a *p*H-stat (using 0.0193 M KOH). At zero time chymotrypsin was added to a final concentration of 0.043 mg/ml; after 24 minutes the temperature was raised rapidly to 43.6° (the thermostatting bath reached the higher temperature in approximately 2 minutes). During the reaction the moles of base per mole of protein required to maintain constant *p*H and the increase in ninhydrin color were measured. Figure 1 shows the base consumption as a function of time of reaction. Parallel changes were observed in the ninhydrin color; by the end of the reaction there was a 61% increase, indicating appreciable peptide bond cleavage (there is an approximately 10% increase in ninhydrin color for each new amino group, a value estimated from the color developed by a mole of leucine relative to that by a mole of ribonuclease A). It is noteworthy that the strong influence of temperature on the reaction occurred precisely where the thermal transition is first apparent (Hermans and Scheraga, 1961a), *i.e.*, at 40 to 45°. Other experiments demonstrated that the rate of hydrolysis is at least 100 times faster at 50° than at 25°, indicating an energy of activation of 35,000 cal./mole, which is about three times that observed for the hydrolysis of bonds in small peptides, and which suggests that conformational changes in the protein are contributing to the activation energy. Unfortunately, rate data could not be obtained at 60° owing to the rapid inactivation of chymotrypsin at this temperature. It should be noted again that, in order to obtain appreciable extents of hydrolysis at 60°, several successive additions of chymotrypsin were made.

An interesting effect of phosphate buffer was observed. The rate of chymotryptic hydrolysis at 50°, *p*H 6.5, was identical in the presence of 0.01 M KCl, 0.2 M KCl, and 0.01 M KCl plus 0.01 M $CaCl_2$. A decrease in rate to 50% of this value was observed in the presence of 0.1 M potassium phosphate buffer, 0.01 M KCl, *p*H 6.5; similar effects were observed in experiments with citrate buffers.

Chromatographic Separation of the Products of Chymotryptic Digestion of Ribonuclease.—The gradient conditions routinely used for chromatography on IRC-50 (see Methods) were chosen to produce optimum separation of high-molecular-weight intermediates in the digestion mixture. However, good resolution of the components of crystalline ribonuclease was also obtained under these conditions, as shown in Figure 2. In chromatography of crystalline ribonuclease there were two inactive lead components, followed by five peaks exhibiting ribonuclease activity, representing 1.5, 7.5, 79.2, 2.7, and 2.2%, respectively, of the total ninhydrin color. Ribonuclease A, obtained by chromatography of the crystalline material in 0.2 M sodium phosphate buffer, *p*H 6.47 (conditions used by Hirs *et al.*, 1953), showed, upon rechromatography under our gradient conditions, principally the fifth component above (designated ribonuclease A in the discussion below), together with substantially undiminished but still small amounts of peaks 6 and 7, and trace amounts of the fast-moving components (1–4). Ribonuclease A eluted after about 3 of the 6 hours of analysis; ammonium ion, applied to the column as ammonium sulfate, eluted after 5.5 hours of analysis.

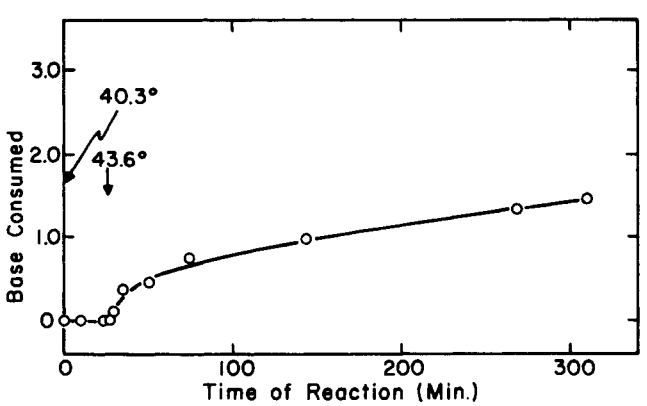

FIG. 1.—The effect of temperature on the rate of chymotryptic hydrolysis of ribonuclease A. The moles of base per mole of protein required to maintain constant *p*H are shown as a function of time. The temperature of the reaction mixture was initially held at 40.3°; after 20 minutes it was raised rapidly to 43.6°.

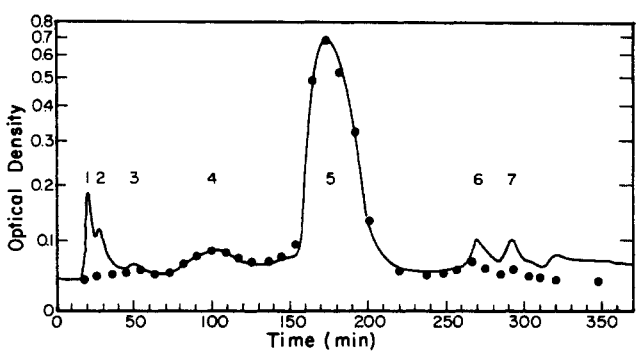

FIG. 2.—The chromatographic pattern from an analysis on IRC-50 of crystalline ribonuclease (Sigma lot R21B-68) under the conditions described in the text. The solid line represents the ninhydrin color of the effluent, continuously monitored with the Technicon Autoanalyzer; the solid circles represent the enzymic activity of the effluent fractions, in arbitrary units so chosen that the maximum enzymic activity and maximum ninhydrin color have the same value.

Figure 3 shows the chromatographic patterns obtained in the analysis of solutions of ribonuclease A digested with chymotrypsin at 60° in the presence of 0.01 M KCl, *p*H 6.5, until slight (10% increase in ninhydrin color), moderate (40% increase), and exhaustive (195% increase) reaction had occurred. After slight digestion the principal component of the reaction mixture was ribonuclease "A" (the quotation marks differentiate the material moving in the position of ribonuclease A, and assumed to be undigested material, from authentic ribonuclease A—see below for indications that it probably *is* principally ribonuclease A). After moderate digestion the quantities of slow and fast-moving components increased at the expense of ribonuclease "A," while after exhaustive digestion the only fractions which remained were the several fast-moving and one slow-moving component (of low molecular weight[1] as demonstrated by gel filtration on Sephadex G-75). The increase and subsequent decrease in the quantity of slow-moving components clearly indicated that there are intermediates in the digestion reaction. A preliminary estimate of the relative sizes of the several components in a medium digest was made by analysis on IRC-50 of the heavy fraction (*i.e.*, all that material which eluted from the Sephadex ahead of the fraction exhibiting maximum ribonuclease activity) obtained by gel filtration of a digestion mixture on Sephadex G-75. The results, shown in Figure 4, demonstrate that ribonuclease "A" and the slow-moving intermediates observed in chromatographic analysis of the unfractionated digest are the only fractions of appreciable size in the digest. A high ratio of ninhydrin color to optical density in the lead fractions of the whole digest confirmed the presence of low-molecular-weight material in them. For reference in the discussion below, the large intermediates in the medium digest (obtained by chromatography on IRC-50) will be designated components KCl-I, -II, -III, and -IV, as shown in Figure 3. KCl-I and -III are the leading and following shoulders on KCl-II, and KCl-IV represents what is certainly a mixture of several components, comprising those fractions from KCl-III to the end of the chromatogram. As can be seen in Figure 3, fraction KCl-II is present in largest amount and, as will be shown below, is of relatively high molecular weight. It is of importance that ribonuclease activity was present only in the ribonuclease "A" peak (KCl-"A"), *i.e.*, the chymotryptic modifications were enzymically inactive.

Changes in Solvent Composition Which Affect the Components Present in Chymotryptic Digests.—Figure 5 presents the chromatographic patterns resulting from the chymotryptic digestion of ribonuclease in the presence of several salts. Digestion at 60°, *p*H 6.5 (under these conditions ribonuclease is about 30% unfolded), in the presence of 0.01 M KCl, 0.2 M KCl, and 0.01 M $CaCl_2$ resulted in the formation of similar products, with particular reference to the large intermediates; however, the relative quantities of these which were produced varied slightly depending upon the conditions of reaction, *e.g.*, in 0.2 M KCl the reaction proceeded more cleanly to components I and II than in 0.01 M KCl. Digestion with chymotrypsin at 25° or 50° (where ribonuclease is only 5% unfolded) in the presence of KCl or $CaCl_2$, or at 60° in the presence of 0.1 M phosphate, yielded only low-molecular-weight products; it is worthy of note that these products were similar to those produced after exhaustive digestion at 60° in the presence of KCl or $CaCl_2$ (see Fig. 3 for a representative pattern).

[1] A consideration of only the fourteen chymotryptic splits which have been located in oxidized ribonuclease (Hirs *et al.*, 1960) shows that after exhaustive proteolysis ten of the twelve possible products would have a molecular weight of about 1000 or less, and the remaining two molecular weights of 2840 and 4270. This is in agreement with the absence of high-molecular-weight material after long reaction.

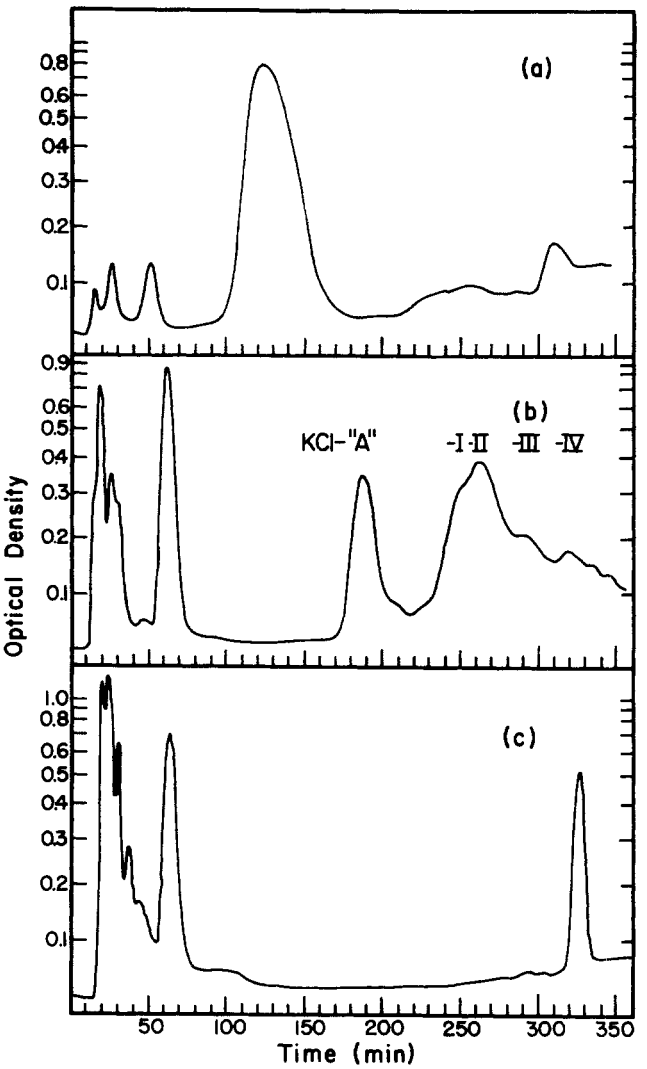

FIG. 3.—The chromatographic patterns from analyses on IRC-50 of ribonuclease A digested with chymotrypsin at 60°, *p*H 6.5, in the presence of 0.01 M KCl: (*a*) slight reaction (10% increase in ninhydrin color); (*b*) moderate reaction (40% increase in ninhydrin color); (*c*) exhaustive reaction (195% increase in ninhydrin color). The ninhydrin color of the effluent in each analysis was continuously monitored, and is shown as a function of the time of analysis.

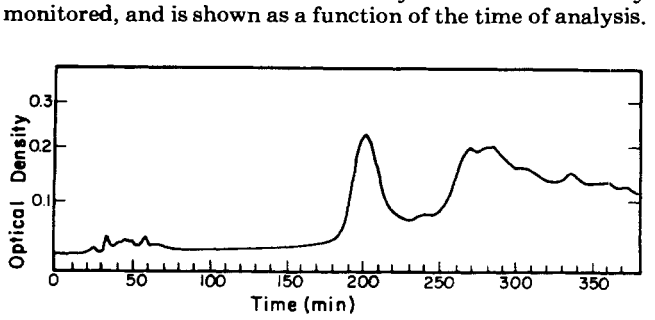

FIG. 4.—The chromatographic pattern from the analysis on IRC-50 of the large components produced by the moderate chymotryptic digestion of ribonuclease A at 60°, *p*H 6.5, 0.01 M KCl. This fraction was obtained by gel filtration on Sephadex G-75. The ninhydrin color of the effluent was continuously monitored, and is shown as a function of the time of analysis.

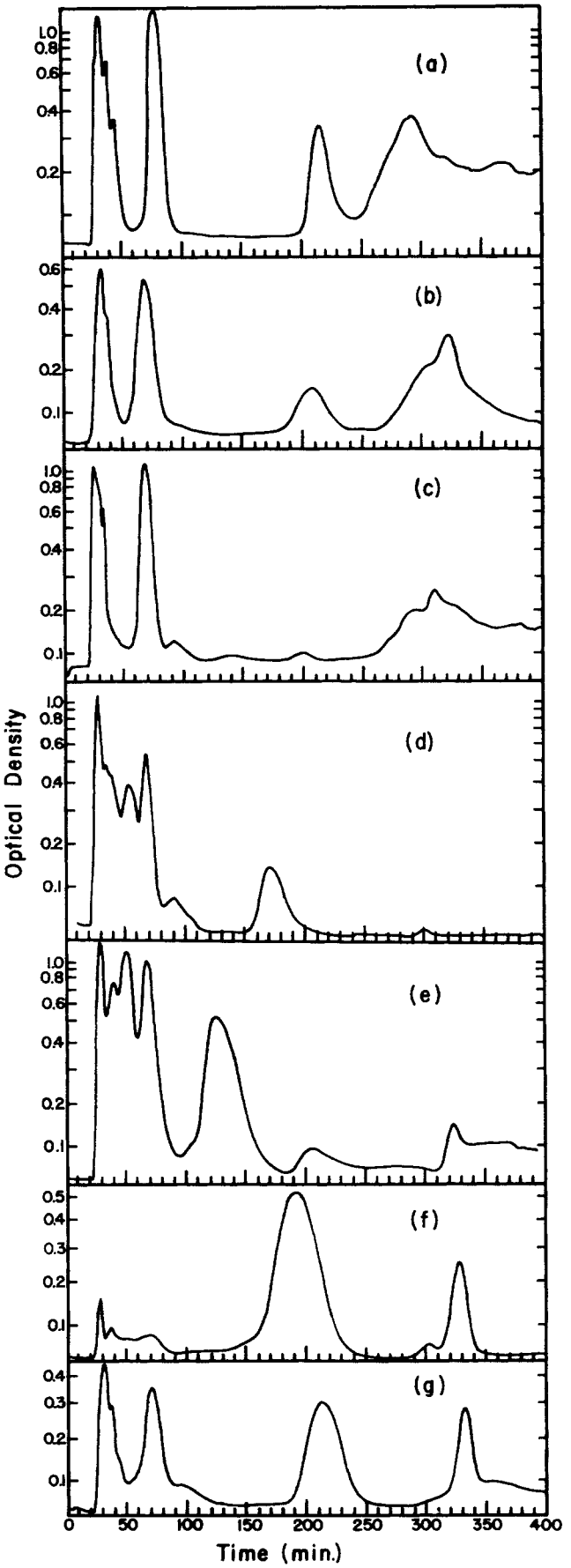

FIG. 5.—Chromatographic patterns from IRC-50 analyses of chymotryptic digests of ribonuclease, obtained at *p*H 6.5 under the following conditions (the % increase in ninhydrin color during the reaction is shown in parentheses): (*a*) 0.01 M KCl, 60° (25%); (*b*) 0.2 M KCl, 60° (70%); (*c*) 0.01 M $CaCl_2$, 60° (105%); (*d*) 0.01 M KCl, 50° (100%); (*e*) 0.01 M $CaCl_2$, 50° (115%); (*f*) 0.01 M KCl, 25° (35%); (*g*) 0.1 M potassium phosphate, 0.01 M KCl, 60° (60%). The ninhydrin color of the effluent in each analysis was continuously monitored, and is shown as a function of the time of analysis.

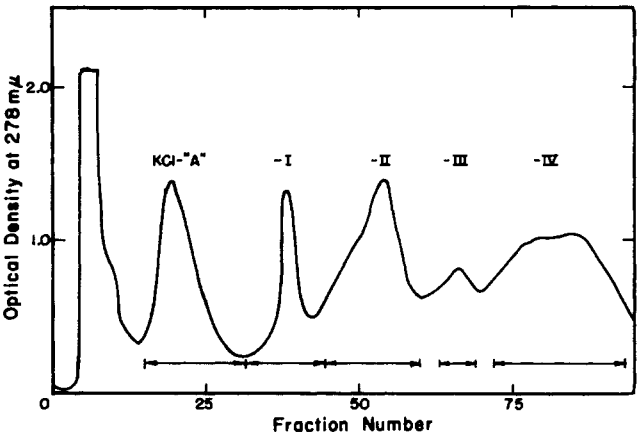

FIG. 6.—Chromatographic pattern of a preparative separation on IRC-50 of a chymotryptic digest of ribonuclease A (60°, 0.01 M KCl, *p*H 6.5). The optical density of the effluent is shown as a function of the fraction number. The arrows above the abscissa indicate the fractions combined to obtain the designated components.

Isolation and Properties of Intermediates in the Digestion Reaction.—As a result of the preliminary information described, the following conditions were chosen to obtain a high yield of the large intermediate KCl-II: 1.8 g of ribonuclease A was digested with chymotrypsin at 60°, 0.01 M KCl, *p*H 6.5, to an increase in ninhydrin color of 25%. After concentration by lyophilization, the reaction mixture was chromatographed on a 1.8 × 43 cm column of IRC-50. The gradient curve was the same as in analytical runs (0.9 × 30 cm columns), but 300 instead of 35 ml of the corresponding buffers was used in each Autograd cylinder. Figure 6 presents the optical density at 278 mμ of the effluent fractions and demonstrates that better resolution was obtained with the preparative than with the analytical column, perhaps owing to the flatter gradient and the 50% longer time of chromatography used with the former. Fractions were combined as indicated in Figure 6 and desalted on a 1.8 × 60 cm column of Sephadex G-25. The yields were as follows: KCl-"A," approximately 160 mg; KCl-I, 88 mg; KCl-II, 244 mg; KCl-III, 75 mg; KCl-IV, 295 mg. Figure 7 presents the effluent patterns obtained from analytical separations of the whole digest and the several fractions obtained from it. KCl-"A," the only material with significant enzymic activity, showed a slow-moving peak at the position of ammonium ion, perhaps residual solvent; KCl-IV was clearly a mixture of several components; KCl-II showed a slow-moving shoulder representing 8% of the total area; KCl-I and -III moved as single peaks. It should be noted that the various fractions after isolation moved on IRC-50 in a manner similar to the corresponding fraction of the whole digest, in that the pattern obtained with the whole digest can be obtained by superposition of those obtained with the various fractions.

Ribonuclease was digested with chymotrypsin as above except that 0.01 M $CaCl_2$ was substituted for 0.01 M KCl. Confirming the results in the preceding section, the pattern obtained in preparative chromatography of the digest showed different relative amounts

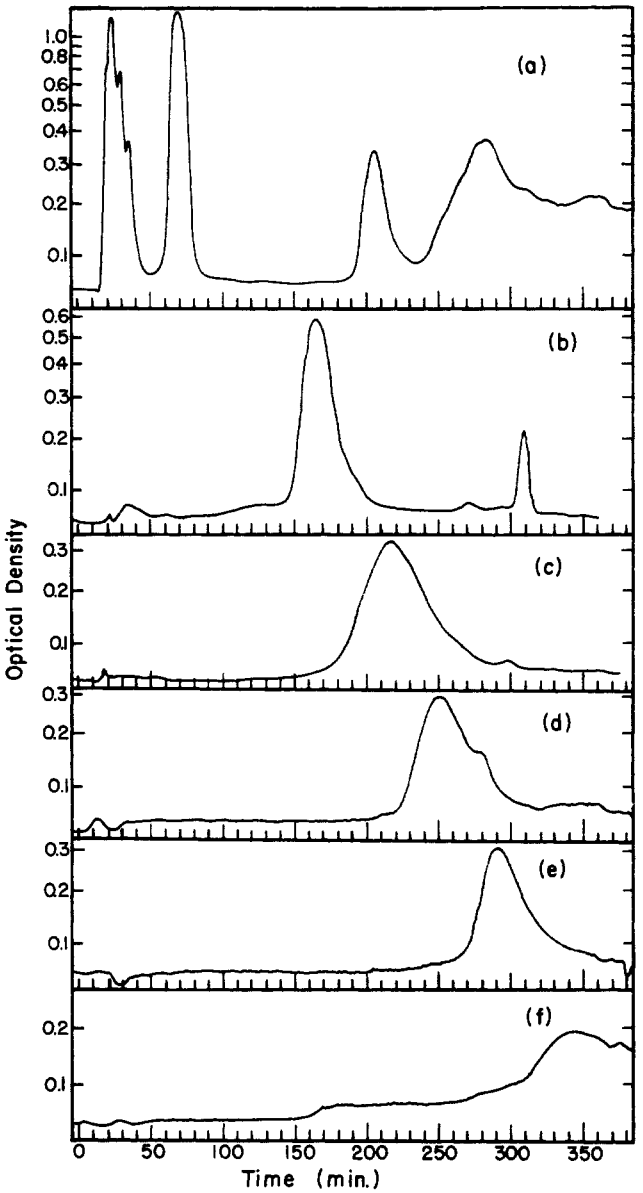

FIG. 7.—Chromatographic patterns of IRC-50 analyses of a chymotryptic digest (0.01 M KCl, 60°, *p*H 6.5) of ribonuclease A and the various fractions isolated from it; the digest and fractions are those shown in Figure 6: (*a*) unfractionated digest; (*b*) KCl-A; (*c*) KCl-I; (*d*) KCl-II; (*e*) KCl-III; (*f*) KCl-IV. The ninhydrin color of the effluent in each analysis was continuously monitored, and is shown as a function of the time of analysis.

of the several intermediates. Two of these, which corresponded chromatographically to KCl-II and to the enzymically active material, $CaCl_2$-"A," were isolated from the digest; both moved as single peaks upon rechromatography.

A tentative estimate of the size of the intermediates was obtained from their sedimentation coefficients, shown in Table I. All moved as single peaks in the ultracentrifuge. A value of $s_{20,w}$ = 1.3 S characterized KCl-I and -II, while $s_{20,w}$ for KCl-IV was considerably higher. The sedimentation coefficient for KCl-III was slightly greater than those for KCl-I and -II, perhaps reflecting contamination by KCl-IV. The $s_{20,w}$ values obtained for KCl-I and -II are in agreement with a molecular weight of about 10,000; the higher value for KCl-IV would be expected for a molecular weight about 17,000, which indicates that KCl-IV is at least in part an aggregate. Ribonuclease A and its derivatives polymerize under certain conditions

TABLE I

SEDIMENTATION COEFFICIENTS OF COMPONENTS OF CHYMOTRYPTIC DIGESTS

Protein	$s_{20,w}$ (S)
KCl-I	1.31
KCl-II	1.29
KCl-III	1.42
KCl-IV	2.18
$CaCl_2$-I	1.27
A	1.80

(Crestfield, 1961; Singer and Richards, 1959); when aggregated, ribonuclease is strongly retarded on IRC-50 (Stein, private communication), in agreement with the behavior observed for KCl-IV. $CaCl_2$-I moved similarly to KCl-I and -II in the ultracentrifuge.[2]

Table II presents several spectral properties of KCl-II, the most plentiful of the modified forms and the one studied in greatest detail. The spectral differences between ribonuclease A and KCl-II demonstrate clearly that there is a normalization of one or more tyrosines during the chymotryptic modification of ribonuclease A. It is noteworthy that the difference spectrum obtained for ribonuclease A between a temperature (71°) at which the thermal transition is nearly complete and room temperature was not observed in KCl-II; indeed a small inverted difference spectrum was present (perhaps owing to nonspecific solvent effects). Since the difference between the temperature difference spectrum observed for ribonuclease A and KCl-II at 71° [0.080 − (−0.250)] is close to the spectral difference between the two derivatives at 25° ($\Delta D_{287m\mu}$ = 0.320), it is a reasonable speculation that the tyrosines normalized in KCl-II are the same as those two tyrosines normalized in the thermal transition. The presence of one abnormal tyrosine in KCl-II

TABLE II

SPECTRAL PROPERTIES OF KCl-II

Ribonuclease A or KCl-II solutions, 3.65 mg/ml, in 0.1 M HCl or 0.02 M potassium phosphate buffer, *p*H 6.5, were prepared; the ionic strength was maintained at 0.2 with KCl. Various difference spectra were then measured at room temperature (25°) unless otherwise noted.

Sample	Reference	$\Delta D_{287m\mu}$
A, *p*H 6.5	A, *p*H 1	+0.100
KCl-II, *p*H 6.5	KCl-II, *p*H 1	+0.040
A, *p*H 6.5	KCl-II, *p*H 6.5	+0.320
A, *p*H 6.5 (71°)	A, *p*H 6.5	−0.250
KCl-II, *p*H 6.5 (71°)	KCl-II, *p*H 6.5	+0.080

is suggested by the appreciable acid difference spectrum found at 25° ($\Delta D_{287m\mu}$ = 0.040). The acid difference spectrum at 25° for ribonuclease A ($\Delta D_{287m\mu}$ = 0.100) contains a contribution from those tyrosines normalized in the thermal transition (and presumably normal in KCl-II); when this contribution is considered the acid difference spectrum (due to one tyrosine) is of a magnitude comparable to that found for KCl-II (Hermans and Scheraga, 1961a,b). In support of the proposal that one tyrosine remains abnormal in KCl-II, the spectral maximum for KCl-II is at 276 mμ, compared with 275 mμ for free tyrosine or oxidized ribonu-

[2] In an earlier experiment a high sedimentation coefficient was obtained for a $CaCl_2$ intermediate (Rupley and Scheraga, 1961), perhaps owing to contamination with material similar to KCl-IV. This led to the erroneous conclusion that $CaCl_2$-I was of a size very near that of ribonuclease A, which in turn led to the interest in the derivatives of $CaCl_2$ as well as KCl.

TABLE III
AMINO ACID ANALYSES OF COMPONENTS OF CHYMOTRYPTIC DIGESTS OF RIBONUCLEASE A
(moles of amino acid/mole of of protein[a])

Amino Acid	Theory A	A	KCl-A	$CaCl_2$-A	Theory A less 1–25	KCl-I	KCl-II	KCl-III	KCl-IV	$CaCl_2$-II[b]
Aspartic acid	15	14.3	15.3	15.6	13	12.6	11.4	12.7	12.1	12.3
Threonine[c]	10	9.5	10.1	10.3	8	7.3	7.1	7.3	7.4	7.4
Serine[c]	15	14.2	14.6	13.6	9	9.2	8.3	8.3	8.3	7.5
Glutamic acid	12	12.5	13.0	13.2	9	9.2	9.3	9.6	9.4	9.5
Proline	4	4.4	4.1	4.1	4	3.6	3.8	3.9	3.4	3.7
Glycine	3	2.9	3.3	3.5	3	2.6	2.7	2.8	3.0	3.1
Alanine	12	11.5	11.0	11.0	7	6.8	6.8	6.6	6.5	6.0
Half-cystine[c]	8	5.9	6.6	7.2	8	6.3	5.6	5.7	6.1	6.3
Valine	9	8.4	9.2	9.7	9	7.7	8.0	8.1	7.9	7.6
Methionine	4	3.8	3.9	4.0	3	2.6	2.3	2.4	2.5	2.2
Isoleucine	3	2.2	2.5	2.8	3	2.1	2.1	2.1	2.2	2.3
Leucine	2	1.9	2.2	2.2	2	1.8	1.8	1.8	2.0	2.1
Tyrosine[c]	6	6.6	6.9	7.0	5	4.9	5.0	5.1	5.3	5.5
Phenylalanine	3	2.9	3.1	2.9	2	1.8	1.7	1.8	1.9	2.0
Lysine	10	10.1	9.8	10.4	8	7.6	7.0	7.6	7.8	7.9
Histidine	4	4.1	4.2	4.1	3	2.8	2.8	2.7	3.0	2.9
Arginine	4	4.0	4.0	4.0	3	2.9	3.0	2.8	3.1	3.2
Molecular weight	13,683				10,922					

[a] The values for moles of amino acid/mole of protein were determined from the experimental values (μmoles of amino acid/sample) by assuming that the μmoles of protein/sample were equal to the average of the μmoles of glutamic acid and alanine/sample divided by their respective theoretical number of residues/molecule of protein. [b] μmoles of protein/sample was determined by averaging the experimental values of μmoles of amino acid/sample divided by the theoretical number of residues/molecule of protein for gly, pro, leu, phe, his, arg. This was done since the alanine value in this analysis seemed unusually low. [c] Correction factors for hydrolysis losses: thr (1.05), ser (1.12), cys/2 (1.23), tyr (1.14), $cySO_3H$ (1.19).

clease A and 277.5 mμ for native ribonuclease A (see Scheraga and Rupley, 1962). Quantitative interpretation of the difference spectra is difficult, owing to the loss (see below) of one fifth of the ribonuclease A molecule, including one tyrosine, in its conversion to KCl-II, and the above comments should be read with this fact in mind.

Amino Acid Analysis of the Reaction Products.—Amino acid analyses were performed on hydrolyzed samples of the various reaction products and on ribonuclease A; the results are shown in Table III. The second column presents the expected moles of amino acid per mole of ribonuclease A. The analyses shown in the next three columns for ribonuclease A, KCl-"A," and $CaCl_2$-"A" agree with theory, generally within 5%, supporting the chromatographic identity between the active component of the chymotryptic digests and the starting material. Some difficulty was experienced in the tyrosine and cystine analyses, perhaps due to different hydrolysis losses than represented by the correction factors applied. The sixth column shows the composition of the molecule of molecular weight 10,922 obtained by deleting the first 25 amino acids from the *N*-terminal tail of ribonuclease A. The amino acid analyses from all the derivatives were nearly identical, and agreed well with the modification just described. The values for serine, threonine, and methionine were generally low (although less than one residue low), and suggest that a peptide, perhaps ser-thr-met (77–79), may have been deleted from a portion of the material constituting each of the chromatographic fractions. This discussion and that which follows are easier to understand through reference to the amino acid sequence of ribonuclease A (Fig. 8).

Isolation and Analysis of the Peptides Resulting from Oxidation of KCl-II.—The peptides obtained from KCl-II by performic acid oxidation of the four disulfide bridges were separated by chromatography on DEAE-cellulose and the several components were obtained by combinations of certain fractions as shown in Figure 9. The components were desalted on Sephadex G-25, and the resulting peptide samples were subjected to amino acid analysis after acid hydrolysis. To interpret the data, obtained as μmoles of amino acid per sample of each component, assumptions must be made; as an example, consider the calculation of the data for component I. The raw data are listed in the second column of Table IV; the notable absence of isoleucine, tyrosine, and histidine, together with a consideration of possible chymotryptic splits, requires that component I be generated from the section of the chain between residues 26 and 46. A second chymotryptic split, between leu 35 and thr 36, is possible in this region of the chain. Peptide 36–46 contains several amino acids (thr, pro, val, phe) not found in peptide 26–35, and the weighted average of the quantities of these amino acids present in the sample determines the quantity of the 36–46 peptide present, *i.e.*, 0.067 μmoles. In turn, the quantities of amino acids not accounted for by peptide 36–46 may be appropriately averaged to determine the amount of peptide 26–35, *i.e.*, 0.252 μmoles. The third column compares the μmoles of each amino acid contributed by the calculated amounts of both peptides with the experimental value.

Table IV also presents the results of similar analyses for all the isolated components of oxidized KCl-II; three fourths of all the calculated values agree with experiment to within 10%, and 90% of the values agree within 20%. In the absence of other information, the selection of the peptides chosen to represent the data for a given component is arbitrary, in that several combinations of chain sections are possible. The selections made assume that the peptides present in any one component are the largest possible, consistent with the continuity of the chromatogram and the charge distribution required by the peptide position in the chromatogram. These indicate chymotryptic splits after residues 25, 35, 46, 76, 79, and 97. Other splits cannot be excluded if they are present in small number, if they yield peptides not isolated, or if they yield two

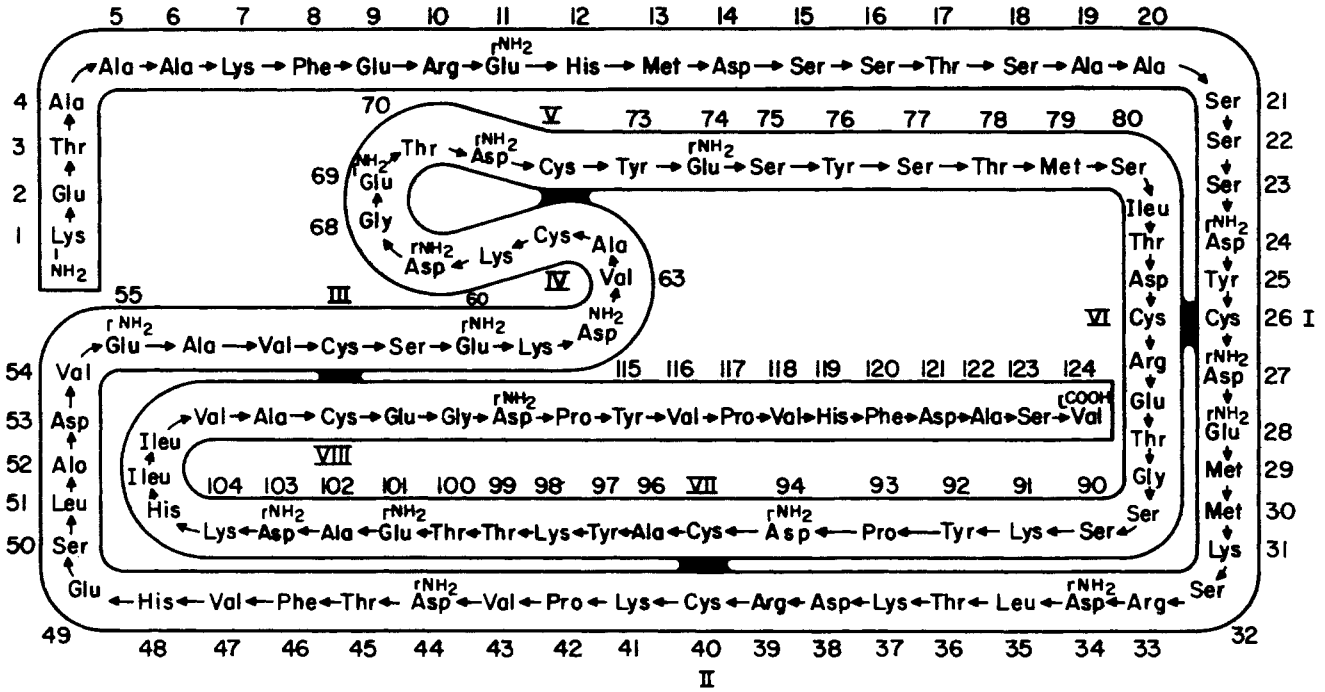

FIG. 8.—The amino acid sequence of ribonuclease A (Hirs *et al.*, 1960; Spackman *et al.*, 1960; Smyth *et al.*, 1962; Potts *et al.*, 1962; Gross and Witkop, 1962; Smyth *et al.*, 1963), adapted from Stein and Moore (1961).

adjacent peptides found in equal amounts in one of the components of oxidized KCl-II. As will be shown below, one additional split is probable.

Analyses for N- and C-Terminal Amino Acids.—The major components of the chymotryptic digest were oxidized with performic acid; both oxidized and unoxidized materials were analyzed for their *N*-terminal groups. Table V presents the *N*-terminal residues found by the DNP method for the intermediates in the chymotryptic digestion of ribonuclease A. The second column indicates that, as expected, lysine is *N*-terminal in ribonuclease A. The small amounts of material listed as "glutamic plus peptide" represents what remained at the origin in the toluene solvent; for ribonuclease A this is an unhydrolyzed lysine peptide (Anfinsen *et al.*, 1954). The amino acids expected to be *N*-terminal in KCl-II are, on the basis of the analyses in the preceding section, cystine, threonine, valine, serine, and lysine. The last four were all found by the DNP method; DNP-cystine and -cysteic acid were not observed, since they were destroyed in the acid hydrolysis. However, a new end-group, DNP-glutamic acid, was identified in the intermediates by rechromatography in phosphate buffer of the spot remaining at the origin in the toluene solvent. The location of the split yielding the group is not clear, but chymotryptic attack at the tyr-glu NH_2 (73–74) bond is clearly possible (Hirs *et al.*, 1960) and reasonable in view of other splits in this region. The low recovery (65%) of *N*-terminal lysine from ribonuclease A indicates that uncorrected losses occurred in the analytical procedure, perhaps owing to the small amounts of material (5 mg) analyzed. Less than one equivalent was found for all end-groups in KCl-II, and, although losses similar to those for ribonuclease A most probably occurred, they would need to be variable and significantly larger to account for the small quantities of several DNP-amino acids found. The conclusion is, thus, that KCl-II contains several chymotryptic splits of *varying* frequency, and therefore is heterogeneous. It is of importance that the four KCl-modifications had similar *N*-terminal residues, in spite of their different chromatographic behavior. $CaCl_2$-I exhibited the same *N*-terminal amino acids.

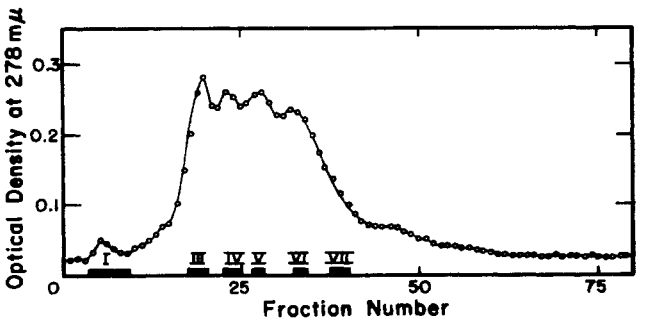

FIG. 9.—The chromatographic pattern from a DEAE-cellulose analysis of performic acid–oxidized KCl-II. The optical density at 278 mμ of the effluent is shown as a function of the fraction number. The solid bars above the abscissa indicate the fractions combined to obtain the corresponding components.

Interestingly, KCl-"A" and $CaCl_2$-"A" contained *N*-terminal groups other than lysine, although in smaller quantities than the inactive derivatives. The possibility exists then that the enzymically active components of the chymotryptic digests might represent modifications of the native molecule. In order to demonstrate the presence or absence of chymotryptic changes in the active components, these were oxidized with performic acid and analyzed on DEAE-cellulose under the conditions employed for the separation of the peptide fragments of KCl-II. Under these conditions oxidized ribonuclease A moved very slowly, with a characteristic pattern emerging after the last peptide fragment. The active components of the digests exhibited largely the oxidized ribonuclease pattern but also contained about one quarter of the total ninhydrin color in peptides characteristic of KCl-II. Similarly the specific activity of the same samples of $CaCl_2$-"A" and KCl-"A" before oxidation were 65 and 81%, respectively, of that of ribonuclease A. Thus the several *N*-terminal groups other than lysine found from the active

TABLE IV
AMINO ACID ANALYSES OF COMPONENTS OF OXIDIZED KCl-II[a]

Amino Acid	Expt. for Component I	Calcd./expt. for {0.067 μmoles 36–46 / 0.252 μmoles 26–35	Expt. for Component III	Calcd./expt. for {0.223 μmoles 26–76 / 0.159 μmoles 98–124	Expt. for Component IV	Calcd./expt. for {0.065 μmoles 26–76 / 0.063 μmoles 36–76 / 0.044 μmoles 36–79	Expt. for Component V	Calcd./expt. for {0.062 μmoles 36–79 / 0.156 μmoles 80–97	Expt. for Component VI	Calcd./expt. for {0.023 μmoles 26–79 / 0.156 μmoles 80–124 / 0.009 μmoles 80–97	Expt. for Component VII	Calcd./expt. for {0.009 μmoles 26–79 / 0.067 μmoles 80–124
Cysteic acid	0.32	0.99	1.29	0.99	0.74	1.03	0.55	1.01	0.62	0.97	0.28	0.89
Aspartic acid	0.51	1.26	2.05	1.10	1.05	1.10	0.64	1.07	0.93	1.05	0.39	1.05
Methionine sulfone	0.56	0.90	0.48	0.94	0.17	1.00	0.11	0.57	0.072	0.96	0.023	1.17
Threonine	0.15	0.91	0.98	1.01	0.53	1.06	0.54	1.04	0.72	1.02	0.30	1.00
Serine	0.29	0.86	1.09	0.96	0.66	0.96	0.68	1.16	0.78	0.98	0.32	0.99
Glutamic acid	0.29	0.86	1.66	1.00	0.95	0.97	0.47	0.99	0.70	0.88	0.28	0.91
Proline	0.058	1.16	0.48	1.12	0.18	0.96	0.21	1.06	0.46	1.08	0.18	1.17
Glycine	0.015	—	0.39	0.98	0.19	0.90	0.23	0.96	0.36	0.96	0.15	0.93
Alanine	0.036	—	1.04	1.10	0.50	1.04	0.35	0.98	0.69	1.02	0.27	1.07
Valine	0.068	0.99	1.56	1.13	0.75	1.14	0.28	1.12	0.72	1.00	0.29	1.05
Isoleucine	—	—	0.18	1.77	0.032	—	0.18	0.85	0.34	1.34	0.14	1.45
Leucine	0.19	1.30	0.44	1.01	0.23	1.02	0.068	0.91	0.044	1.05	0.020	0.90
Tyrosine	—	—	0.62	0.98	0.33	1.04	0.43	1.01	0.58	0.91	0.23	0.96
Phenylalanine	0.070	0.96	0.37	1.03	0.16	1.05	0.055	1.12	0.18	1.01	0.075	1.02
Lysine	0.39	0.99	1.55	0.92	0.77	0.97	0.48	0.84	0.62	0.96	0.29	0.86
Histidine	—	—	0.50	1.08	0.19	0.89	0.101	0.39	0.34	0.99	0.15	0.93
Arginine	0.30	1.06	0.42	1.06	0.22	1.06	0.200	1.09	0.17	1.24	0.078	1.09

[a] Experimental data are given as μmoles of amino acid in the sample analyzed.

TABLE V
DETERMINATION OF *N*-TERMINAL AMINO ACIDS BY DNP METHOD
(Moles DNP-amino acid/mole protein[a])

DNP-Amino Acid	A	KCl-I	KCl-II	KCl-III	KCl-IV	$CaCl_2$-I	KCl-"A"	$CaCl_2$-"A"
Glutamic + peptide	0.04 (0.13)	0.44 (1.34)	0.24 (0.78)	0.29 (0.75)	0.30 (1.12)	0.09	0.23	0.22
Serine	0	0.51 (0.98)	0.42 (0.62)	0.41 (0.49)	0.50 (0.92)	0.34	0.17	0.23
Threonine	0	0.14 (0.27)	0.25 (0.29)	0.22 (0.24)	0.24 (0.49)	0.26	0.11	0.21
Valine	0	0.18 (0.20)	0.10 (0.10)	0.11 (0.10)	0.11 (0.15)	0.08	0.07	0.24
Lysine	0.65 (0.70)	0.28 (0.43)	0.44 (0.41)	0.48 (0.53)	0.41 (0.90)	0.57	0.67	0.69

[a] Data in parentheses were obtained by analyses of samples of oxidized protein.

components probably arise from small amounts of inactive intermediates moving with the "A" peaks.

The digestion of KCl-II with leucine-aminopeptidase led to the release of a large number of amino acids, rendering interpretation difficult. However, the six expected *N*-terminal residues, including the cysteic acid not detected by DNP-analysis, were present in appreciable concentrations.

Analysis for the *C*-terminal amino acids of KCl-II was performed by the carboxypeptidase method. The expected *C*-terminal residues (valine, methionine, tyrosine, phenylalanine, and leucine) were present in the digest, together with several others which could be simply accounted for by assuming that adjacent interior amino acids were partially removed in several instances (in particular those comprising the *C*-terminal sequence of ribonuclease A- and KCl-II- through his-119).

DISCUSSION

The Positions of the Chymotryptic Splits.—Amino acid analyses of the intermediates found in chymotryptic digests of ribonuclease A show that in thermally unfolded ribonuclease the tyr-cys (25–26) bond is rapidly split. The analysis of peptide mixtures isolated from oxidized KCl-II determined the probable location of five other sensitive bonds: met-ser (79–80), tyr-lys (97–98), leu-thr (35–36), tyr-ser (76–77), and phe-val (46–47); these are listed in the order of decreasing frequency of splitting, estimated from the DNP analyses and the pattern of the peptides in oxidized KCl-II. End-group analyses served to support the assignments and to determine the presence of one otherwise undefined split, at a bond whose amino portion is contributed by glutamic acid or glutamine.

Relationships Between the Chymotryptic Splits, the Thermal Transition, and Other Properties of Ribonuclease.—Partial unfolding of ribonuclease in the thermal transition may render peptide bonds in the regions unfolded available to chymotryptic hydrolysis, and the resulting splits may be termed primary. However, a further unfolding of the molecule may occur, triggered by the primary splits, and thereafter chymotryptic hydrolysis may lead to secondary splits. Primary sites of hydrolysis are the only ones of interest for locating regions of the polypeptide chain affected in the thermal transition; those hydrolyses occurring with the highest frequency will clearly have the greatest probability of being primary. Since the tyr-cys (25–26) bond is quantitatively split in all the intermediates isolated,

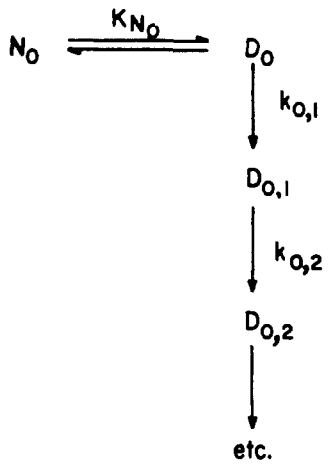

FIG. 10.—A kinetic scheme describing the chymotryptic hydrolysis of ribonuclease A.

the region of the chain around the first disulfide bond can with confidence be said to unfold in the thermal transition. Other evidence supports this conclusion. Trypsin hydrolyzes thermally unfolded ribonuclease at the bonds lys-ser (31–32) and arg-aspNH_2 (33–34) (Ooi *et al.*, 1963). Richards and Vithayathil (1959) have shown that the ala-ser (20–21) bond is accessible to subtilisin attack in the native molecule, indicating that this portion of the chain is accessible in the native protein, although a conformational change is required to render it accessible to chymotrypsin. Since arg-aspNH_2 (33–34) is the site of a primary tryptic split, the chymotryptic hydrolysis at leu-thr (35–36) probably is also a direct result of the thermal unfolding, and the entire region comprising residues 25–36 may unfold in the transition.

Similar considerations lead to the conclusion that the central section of the chain, residues 73–80, also unfolds in the thermal transition. The frequency of the split at met-ser (79–80) indicates that it is primary, suggesting a similar status for that at tyr-ser (76–77). Finally, the DNP-glutamic acid found may result from a split at tyr-gluNH_2 (73–74).

The data indicate that tyr-lys (97–98) is attacked by chymotrypsin, while tyr-val (115–116) is not. Since *N*-terminal lysine residues were found in large quantity, the region of the chain including tyrosine 97[3] must unfold in the thermal transition, while that surrounding tyrosine 115 is unaffected. The conclusion that the thermal transition does not affect the *C*-terminal tail is supported by the failure of chymotrypsin to split three other bonds in the *C*-terminal portion of thermally unfolded ribonuclease, which are split in oxidized ribonuclease A [gluNH_2-lys (103–104), his-ileu (105–106), and phe-asp (120–121); Hirs *et al.*, 1960]. It is of interest that the *N*-terminal tail is similarly unaffected in the thermal transition (Ooi *et al.*, 1963), in view of the probable structural interrelationship between the *N*- and *C*-terminal regions (see discussion in Scheraga and Rupley, 1962). The presence of structure around tyrosine 115 suffices to explain the presence of an acid *p*H difference spectrum in KCl-II, for this presumably results from the removal of this remaining abnormality. A specific unfolding of the *C*-terminus of ribonuclease at low *p*H agrees with the proposal that the abnormal tyrosine affected at low *p*H is not one of the two involved in the thermal transition (Bigelow and Sonnenberg, 1962), and also explains why at low *p*H pepsin readily cleaves the phe-asp (120–121) bond (Anfinsen, 1956), while at neutral *p*H and at elevated temperature chymotrypsin, with a specificity for phenylalanyl bonds, does not. The present data are inadequate to determine whether the hydrolysis of the bond phe-val (46–47) locates a region of unfolding or is a secondary split.

The failure of ribonuclease to refold upon reduction of the temperature[4] after chymotryptic hydrolysis, as well as the spectral changes and the loss of the *N*-terminal tail, means that one or more of the bonds hydrolyzed is in a region involved in the maintenance of the structure of the native molecule. Since splits can occur at bonds 20–21, 31–32, and 33–34 without complete loss of enzymic activity or the *N*-terminal tail, the cleavage at bonds 25–26 probably is not critical. Of the two remaining regions where primary splits occur, 73–80 and 92–97, the elucidation of which (if either) is more critical for the structural integrity of ribonuclease may follow the determination of which tyrosines in ribonuclease are abnormal.

It is not entirely clear how KCl-I, -II, -III, and -IV can possess nearly identical amino acid and end-group compositions and yet move differently on IRC-50. In preliminary experiments, differences in the peptide patterns obtained from the oxidized intermediates were observed. These differences were in the relative proportions rather than in the types of peptides present, perhaps indicating different relative frequencies of the several bonds split in each of the four components. Alternatively, the rate of disulfide interchange is appreciable at 60° and neutral *p*H (Spackman *et al.*, 1960), and it may be relevant to the observed chromatographic differences and the aggregation found for component KCl-IV. Also, it must be noted that none of the isolated intermediates can be homogeneous, for the different frequency of the several splits in any one component (*e.g.*, KCl-II) requires that it comprise several different although probably closely related molecules.

The Effects of Solvents and Temperature on the Chymotryptic Digestion.—Only low-molecular-weight products were obtained when chymotryptic digestion was performed at 25° or 50°, in contrast to the large intermediates found at 60°. Also, at 60° the rate of digestion was significantly reduced in the presence of phosphate and citrate ions, and the products obtained in the presence of phosphate (no investigation was made for citrate) were all of low molecular weight. Finally, at 60°, the presence of calcium ion or a higher ionic strength led to a slightly different distribution among the various large intermediates.

These results may be made reasonable by consideration of a kinetic scheme (Fig. 10) similar to one proposed by Green and Neurath (1954) and based on the discussion of Linderstrøm-Lang (1952). In this scheme there is an equilibrium between native (N_0) and denatured (D_0) forms with an equilibrium constant K_{N_0}. The concentration of D_0, but not its structure, is assumed to be temperature dependent. The species D_0 can be hydrolyzed step-wise at rates characterized by the constants $k_{0,i}$; chymotrypsin does not attack the native molecule (N_0) rapidly (Spackman *et al.*, 1960). The intermediate $D_{0,1}$, of high molecular weight, will accumulate if $k_{0,2}$ is small compared to $k_{0,1}$. If it is assumed that an increase in temperature increases K_{N_0} more rapidly than the constants $k_{0,i}$,

[3] The tyr-pro (92–93) bond is not susceptible to chymotryptic hydrolysis, even in oxidized ribonuclease (Hirs *et al.*, 1960), and this portion of the chain may consequently be involved as well in the transition.

[4] Native ribonuclease is *reversibly* and partially unfolded at 60°.

etc., $D_{0,1}$ will accumulate, as is observed. Since there are several intermediates of high molecular weight ($D_{0,1}$, $D_{0,2}$, *etc.*), an effect of ionic strength or calcium ion upon the several respective rate constants would affect the distribution of the intermediates (for simplicity, parallel paths are not considered). Finally the effect of phosphate or citrate may result from a decrease in the equilibrium constant K_{N_0}, or a decrease in $k_{0,1}$ relative to $k_{0,i}$, a rate constant representing degradation of a large intermediate to low-molecular-weight products. There is evidence (Sela *et al.*, 1957; Sela and Anfinsen, 1957) that phosphate stabilizes a folded form of ribonuclease (an effect on K_{N_0}); however, the same effect was not observed for citrate ions.

Similar considerations can be applied (Scheraga and Rupley, 1962) to the proteolysis of ribonuclease by subtilisin and pepsin.

Acknowledgment

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