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The Action of Trypsin on Ribonuclease-S*

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S-Protein and *S*-peptide, either separately or combined in the active enzyme RNase-*S*, can be digested by trypsin, whereas the native enzyme, RNase-*A*, is not attacked. In *S*-peptide the bond between residues 10 and 11 is hydrolyzed much more rapidly than the one between numbers 7 and 8. Cleavage of the first bond results in a loss of all demonstrable interaction with *S*-protein. Residue 11 appears to be a glutamyl (or glutaminyl) group, in agreement with the results of White and Anfinsen (1959). The tryptic attack on *S*-protein produces at least three chromatographically separable intermediates which all bind *S*-peptide and produce complexes with varying catalytic activity but in all cases less than that observed with *S*-protein itself. These intermediates appear to contain most, if not all, of the residues present in *S*-protein, but additional amino-terminal groups are found. The maximum number of bonds hydrolyzed in *S*-peptide is 2, in *S*-protein 7 to 8, but in RNase-*S* only 6 to 7. From this discrepancy and the kinetics of the activity loss, it is concluded that trypsin can attack RNase-*S* without dissociation of the parts, but that the course of proteolysis and the final products are not the same as in the sum of the digests of the separated components. *S*-Protein polymerizes in the neutral *p*H range but appears to be a monomer at *p*H 2, with a sharp transition between *p*H 2 and 3. The polymers are dissociated easily by *S*-peptide. The complex, RNase-*S*, does not show any tendency to polymerize. No evidence for polymerization of the tryptic digestion intermediates was found. In the very early stages of digestion of *S*-protein and RNase-*S* by trypsin, an increase in the RNase activity was observed. This was not seen with *S*-peptide or RNase-*A*. No correlation was established with extent of hydrolysis or amount of trypsin. Chromatograms of partial digests have not shown any material with specific activity higher than the starting material and the activity recoveries were low. No explanation has been found for this activation effect.

Proteolytic enzymes vary widely in their ability to attack "native" protein substrates, with the plant enzymes in general being much less fastidious than those of animal origin. In almost all cases the rate of proteolysis is increased by prior denaturation of the substrate. In earlier studies such denaturation was usually effected by exposure of the protein to high temperatures or extremes of *p*H, resulting in gross changes in physical properties (Green and Neurath, 1954). More recently it has been noted in many studies that altered susceptibility of enzymes used as substrates occurs in the presence or absence of their activators, coenzymes, or competitive inhibitors (see, for example, Fischer and Stein, 1960). The extent of the structural change in a substrate protein required to effect digestion by a proteolytic enzyme may be very subtle indeed. Such a case is reported in this paper.

Ribonuclease-*S* is produced from the native bovine pancreatic enzyme by cleavage of the peptide bond between residues 20 and 21 (Richards and Vitayathil, 1959). In dilute aqueous buffer solutions in the *p*H range 5 to 9 this modified enzyme is qualitatively and quantitatively indistinguishable from the native enzyme: in its catalytic activity toward a variety of substrates; in its ability to precipitate antisera against the latter (Singer and Richards, 1959); and in its sedimenta-

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tion, electrophoretic, and optical rotatory behavior. Clearly the structural change which must have occurred can have affected only a small part of the molecule at some distance from the catalytic site. The most dramatic difference between the two enzymes is shown by the influence of trypsin and chymotrypsin (Richards, 1958). These two pancreatic proteinases are essentially without effect on native ribonuclease at neutral *p*H and room temperature. However, RNase-*S*[1] is rapidly digested under these conditions. Some observations on the tryptic digestion are given below.

Experimental Procedure and Results

The preparation of RNase-*A*, RNase-*S*, *S*-protein, and *S*-peptide, the activity assay procedures, and the procedures for amino acid analysis and paper electrophoresis have been described (Richards and Vithayathil, 1959; Vithayathil and Richards, 1960). Commercial samples from Armour Inc., Lot 381-059, and Sigma Laboratories, Lot. No. R99-80, were used as starting materials for the protein preparations.

A trypsin preparation containing 50% by weight of $MgSO_4$ was kindly donated by Dr. J. S. Fruton. No ammonia release in excess of the blank value was observed when this preparation was incubated with glycyl-L-tyrosine amide according to the procedure of Laskowksi (1955). The residual "chymotryptic" activity of the trypsin itself towards this substrate was within the error of the assay as performed (Inagami and Sturtevant, 1960; Cole and Kinkade, 1961).

Total Number of Bonds Split by Trypsin in Various Protein and Peptide Preparations.—The digestions were carried out in a *p*H-stat (Radiometer Inc. Model TTTla) at 25° under a nitrogen atmosphere. The *p*H was maintained at 8.0 by the addition of standard alkali. The reactions were allowed to run until no further uptake of alkali occurred for more than an hour. Additional trypsin was then added. If no further digestion occurred, the reaction was assumed to have gone to completion.

The base uptake per mole of protein was multiplied by the factor 1.09 to obtain an estimate of the number of bonds hydrolyzed. This factor was obtained from the data on performic acid–oxidized RNase-*A* where the average base uptake was assumed to correspond to 12 bonds per mole on the basis of the study by Hirs *et al.* (1956). It is equivalent to assuming an average pK' value of about 7 for the amino groups produced during hydrolysis, and corresponds closely to those found in other similar studies (Richards, 1955). The results obtained on several preparations are listed in Table I.

[1] The following abbreviations are used: RNase-*A*, the principal chromatographic component of bovine pancreatic ribonuclease; RNase-*S*, subtilisin-modified ribonuclease; *S*-peptide, the 20-residue peptide component obtained from RNase-*S*; *S*-protein, the protein component obtained from RNase-*S*; RNase-*S'*, the reconstituted enzyme obtained by mixing equimolar amounts of *S*-peptide and *S*-protein.

Table I

Total Number of Peptide Bonds Split During Tryptic Hydrolysis

The indicated weight of the substrate was dissolved in 10 ml of water in the *p*H-stat cell at 25°. The *p*H was adjusted to 8.0 with 0.1 *N* HCl and was maintained at that value by the addition of 0.025 M NaOH.

Substrate (mg)		Trypsin (mg)	Total Base Uptake (μmoles)	Total Bonds Cleaved per Mole of Substrate[a]		Maximum No. of Bonds Susceptible Trypsin[b]
Oxidized RNase-*A*	6.1	0.5	4.9	12.3	12.0	12
	5.0	0.5	3.8	11.7		
RNase-*S*	7.0	0.1	3.0	6.4	6.8	12
	7.0	0.1	3.4	7.2		
RNase-*S'*	6.7	0.1	2.8	6.2	6.6	12
	6.7	0.1	3.1	6.9		
S-protein	6.6	0.5	4.1	7.8	7.7	10
	6.6	0.1	3.8	7.2		
	6.0	0.15	3.9	8.1		
S-peptide	16.0	0.06	13.5	2.03		2

[a] The figures were calculated by multiplying by 1.09 (see text) the moles of base per mole of protein. For the latter number the following molecular weights were assumed: Oxidized RNase-*A* 14,100; RNase-*S* and RNase-*S'* 13,700; *S*-protein 11,500; and *S*-peptide 2,300. [b] See Hirs *et al.* (1956).

The Action of Trypsin on S-Peptide.—It was expected that the bonds involving the lysyl residue (no. 7) and the arginyl residue (no. 10) would be cleaved, but not the one next to the amino-terminal lysyl residue (no. 1). The course of the digestion of a sample of *S*-peptide is shown in Figure 1. From the marked change in slope after about one half the maximum base uptake, it is clear that one of the two bonds is split much more rapidly than the other. The rapid loss in activity shown by the digest would indicate that the splitting of this bond in the peptide was sufficient to prevent the formation of a catalytically active complex with

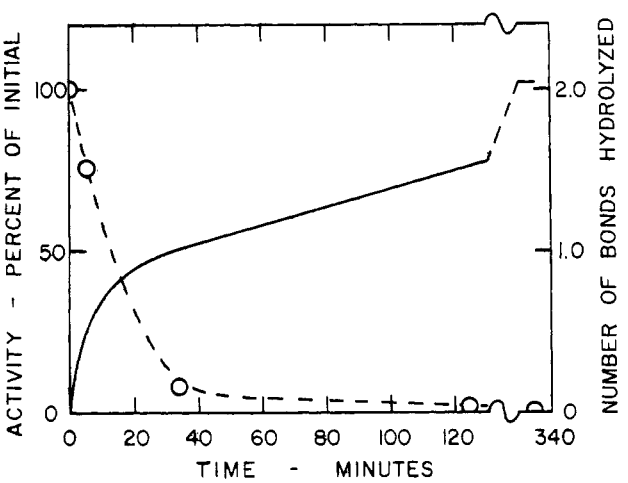

Fig. 1.—Digestion of *S*-peptide by trypsin at *p*H 8 and 25°. Sixteen mg of *S*-peptide and 60 μg of trypsin in 8 ml of water were placed in the *p*H-stat. The solid line is redrawn from the *p*H-stat chart and refers to right hand ordinate converted from volume of base to number of bonds split. The open circles indicate the assay values made in the presence of a slight excess of *S*-protein with RNA as substrate.

S-protein. The final digest, where both bonds were split, not only produced no activity when mixed with *S*-protein even at a tenfold molar excess but also failed to cause any inhibition of the activity produced by equimolar amounts of *S*-protein and *S*-peptide. These observations imply that the three products of the tryptic digestion of *S*-peptide (tested either separately or together in the same solution) are lacking in, or have a greatly reduced, ability to bind to *S*-protein. If the binding constant had been reduced by a factor of less than 100, measurable inhibition should have been observed in the assays performed with ten- to twentyfold excesses of the total digest or its fractions.

Two-dimensional separations of some digests were carried out on paper as shown in Figure 2.

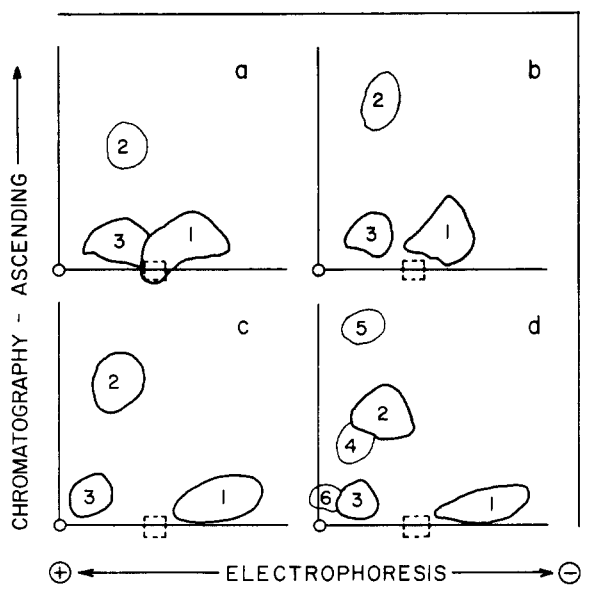

FIG. 2.—Two-dimensional separation of tryptic digests of *S*-peptide. Two mg of *S*-peptide and 40 μg of trypsin in 0.2 ml of 1% lutidine, *p*H 7.8, were allowed to stand at 25°. Aliquots (30 μl) were removed at various times, acidified to stop the tryptic action, assayed, and run on Whatman No. 1 paper. The first dimension was electrophoresis in pyridine acetate buffer, *p*H 6, 20 v/cm, 1½ hours, the second dimension ascending chromatography in 1-butanol–acetic acid–water, 3:1:1. Open circles show the origin; the dashed squares show the position of undigested *S*-peptide estimated from guide strips. *a*, Digestion for 15 minutes, 20% of initial activity remaining; *b*, 30 minutes, activity 0; *c*, 2 hours; *d*, 30 hours.

The spot labeled "2" was very faint in *a*, strong in *b*, and roughly equivalent to 1 and 3 in *c*. Material moving as the undigested peptide was present only in *a*. The changing appearance of spot "1" in the various patterns probably indicates that it represents a mixture of substances. The slow appearance of the spot labeled "2" is in accord with the conclusion drawn from the data in Figure 1. The rapid splitting of the first bond produced the materials labeled "1" and "3," and subsequent splitting of the second bond released the material in spot "2." The activity of the digest has already disappeared when only traces of "2" are apparent. The very long digest, Figure 2*d*, shows the appearance of small additional amounts of peptides presumably caused by traces of impurities in the trypsin sample used.

Preparative scale separations of a larger digest were carried out on paper substantially as above. The eluted materials were hydrolyzed and their amino acid composition determined (Table II). Fraction 2, corresponding to spot 2 in Figure 2, was clearly a pure preparation of the tripeptide (Phe·Glu·Arg). Fraction 1 was apparently a mixture of the amino-terminal heptapeptide expected in a complete digest and the decapeptide resulting from incomplete splitting in this digest of the bond between residues 7 and 8 in the original *S*-peptide. The hepta- and decapeptides have the same net charge and would be expected to move very similarly in electrophoresis. Neither moved far enough in the chromatographic dimension to effect a separation. From these results the bond between residues 10 and 11 would appear to be the first one split and the one whose hydrolysis is responsible for the loss of activity.

The amino acid analysis of fraction 3 isolated after chromatography was very strange. Some amino acids were missing entirely (*i.e.*, glutamic acid), while several unidentifiable peaks appeared in the chromatogram. This same behavior was observed in two separate sets of isolations and analyses. No explanation can be offered, but similar problems have been noticed by others during analysis of paper eluates (D. F. Elliott, personal communication). Material from the electrophoresis step prior to chromatography gave a perfectly satisfactory analysis [Table II, Fraction 3(+2)] after allowance was made for some contamination with fraction 2 which could be demonstrated easily by chromatography or end-group analysis.

The amino-terminal end-groups of these fractions, as determined by the fluorodinitrobenzene procedure, are shown in Table III. The total digest showed only three different amino acids as end-groups: lysine, phenylalanine, and glutamic acid. The phenylalanine clearly belongs to the tripeptide of fraction 2 as expected (Hirs *et al.*, 1960). Fraction 1 contains principally the lysine of the original *S*-peptide (the trace of glutamic acid cannot be explained). Fraction 3(+2) contains the phenylalanine as an impurity, as expected from the amino acid analysis. However, the principal end-group on this last fraction was glutamic acid. No trace of serine as an end-group was found in any of the chromatograms. The sequence reported by Hirs *et al.* (1960) places serine in position 11 of the peptide chain. White and Anfinsen (1959), however, believe that this is a glutamyl residue, in agreement with the results found here. No other evidence bearing on the sequence of residues 11 through 20 was obtained in this study.

Action of Trypsin on S-Protein.—The course of the digestion of *S*-protein is shown in Figure 3.

TABLE II

AMINO ACID ANALYSIS OF FRACTIONS FROM TRYPTIC DIGESTS OF *S*-PEPTIDE AND *S*-PROTEIN

Ten mg of *S*-peptide + 40 μg of typsin were incubated for 5 hr in 0.5 ml of 1% lutidine at *p*H 7.8. The fractions were separated by preparative electrophoresis and chromatography. For the *S*-protein derivative, peak β_2, see text. The symbol *t* indicates that a visible deflection in base line was observed but was too small to quantitate; a zero indicates that there was no detectable variation in base line.

	Residues per mole							
	Fraction 1		Fraction 2		Fraction 3(+2)		Peak β_2	
Amino Acid	Found	Calc.[a]	Found	Calc.[b]	Found	Calc.[c]	Found	Composition of *S*-Protein
Lysine	1.82	2.0	*t*		*t*		7.8	8
Histidine	*t*		*t*		0.98	1.0	2.9	3
Arginine	0.54	0.5	1.05	1.0	0.38	0.3	2.2	3
Aspartic acid	*t*		*t*		1.02	1.0	13.7	14
Threonine	1.00	1.0	*t*		1.01	1.0	7.4[d]	8
Serine	*t*		*t*		2.85	3.0	10.1[d]	12
Glutamic acid	1.53	1.5	0.96	1.0	1.26	1.3	9.0	9
Proline	0		0		0		3.4	4
Glycine	*t*		*t*		*t*		3.2	3
Alanine	3.05	3.0	*t*		2.06	2.0	6.7	7
Cystine/2	0		0		0		6.7[d]	8
Valine	0		0		0		8.5	9
Methionine	0		0		0.99	1.0	2.8	3
Isoleucine	*t*		0		*t*		2.1[d]	3
Leucine	*t*		*t*		*t*		1.8	2
Tyrosine	0		0		0		5.5	6
Phenylalanine	0.48	0.5	1.03	1.0	0.32	0.3	2.2	2
Sample per column μmoles	0.18		0.11		0.20			

[a] *S*-peptide corresponds to the first 20 amino acids in the sequence of RNase-*A* given by Hirs *et al.* (1960). Trypsin splitting would be expected at bonds 7–8 and 10–11 (Hirs *et al.*, 1956). Fraction 1 was assumed to be a 50:50 mixture of the amine-terminal heptapeptide (Lys_2.Thr.Glu.Ala_3) and the decapeptide (Lys_2.Arg.Thr.Glu_2.Ala_3.Phe). [b] See *a*. Fraction 2 was assumed to be the tripeptide (Phe.Glu.Arg) corresponding to residues 8 through 10 in the *S*-peptide sequence. [c] See *a*. Fraction 3 was assumed to be a mixture of the decapeptide (His.Asp.Thr.Ser_3.Glu.Ala_2.Met) (residues 11–20) 77% and the tripeptide (Phe.Glu.Arg) 23%. [d] No corrections made for destruction or incomplete release during hydrolysis.

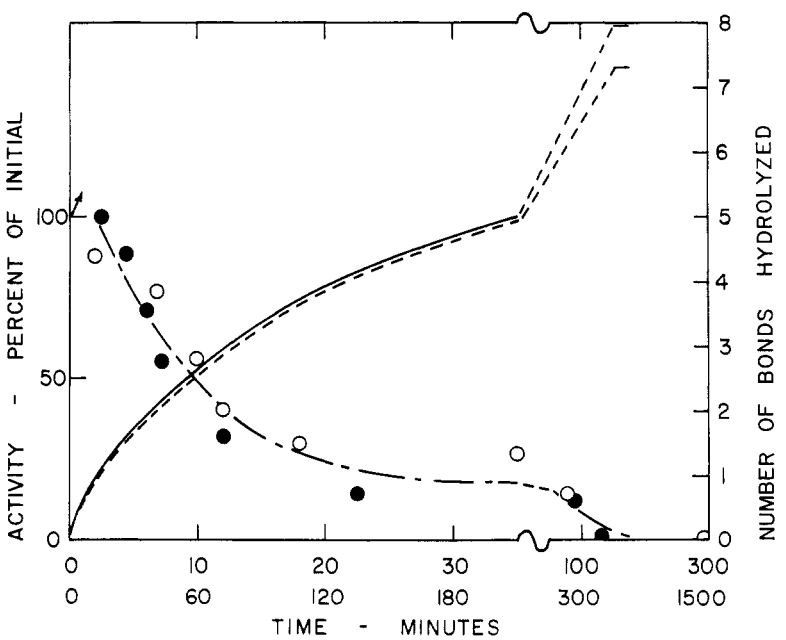

FIG. 3.—Digestion of *S*-protein by trypsin at *p*H 8.0 and 25°. Samples were dissolved in 10 ml of water. The solid line and the open circles refer to the alkali uptake and activity measurements observed during the digestion of 6.6 mg of *S*-protein with 150 μg of trypsin and refer to the upper time scale on the abscissa. The lower time scale refers to the dashed line and filled circles giving data obtained on the digestion of 6.0 mg of *S*-protein with 25 μg of trypsin. The arrow in the diagram refers to the aliquots taken at very early times where activities in excess of 100% were observed and where the extent of hydrolysis was uncertain (see text).

TABLE III

AMINO-TERMINAL END-GROUPS OF SOME FRACTIONS FROM TRYPTIC DIGESTS OF *S*-PEPTIDE AND *S*-PROTEIN

End-groups determined by the fluorodinitrobenzene procedure (Fraenkel-Conrat *et al.*, 1955). All spots visible on the two-dimensional chromatograms are listed. Any substances present to the extent of 5% of the principal ones found would have been easily visible.

Preparation	Dinitrophenyl Amino Acid	Preparation	Dinitrophenyl Amino Acid
S-peptide	Lysine	*S*-protein	Serine
S-peptide total digest	Lysine	Peak β_1	Serine
	Phenylalanine		Aspartic acid (asparagine)
	Glutamic acid		Tyrosine
Fraction 1	Lysine	Peak β_2	Serine
	Glutamic acid (trace)		Aspartic acid (asparagine)
			Tyrosine
			Threonine
Fraction 2	Phenylalanine		
Fraction 3(+2)	Phenylalanine		
	Glutamic acid		

No unique relation between bond splitting and activity loss can be derived from these data alone. A sixfold change in trypsin concentration had no affect on either base uptake or activity loss except to alter the time scale by the same factor. For a given per cent reaction the enzyme-time product is a constant, as might be expected, and this relation can be used to compare the rates of inactivation observed in various separate experiments.

An activation effect was noted in the very early stages of digestion which was not seen at all with *S*-peptide. In every experiment on the tryptic digestion of *S*-protein or RNase-*S* a very rapid rise in activity was seen at the beginning of the digestion (occasionally as much as 30 to 40% above the initial activity measured on an aliquot taken before the addition of trypsin). Some typical data are collected in Table IV. At 25° the largest activation was observed at the earliest times tested. By the time the alkali uptake corresponded to an average splitting of one bond per mole the activity had returned to its initial value. When the *S*-protein and trypsin were mixed at 2°, no detectable hydrolysis occurred but the activation was still noted even though the aliquot taken for assay was at 25° for only a few seconds before mixing with the substrate. The RNase assay was carried out at *p*H 5.0, where trypsin shows little or no proteolytic activity. No activation was noted in mixtures of trypsin and RNase-*A*, nor could any effects of trypsin on the assay procedure be demonstrated. The consistency of the effect both in duplicate assays and between separate experiments would appear to put the activation effects well outside the range of possible experimental error.

Chromatograms on carboxymethylcellulose of some of the starting materials are shown in Figure 4. The separation between RNase-*A* and RNase-*S* did not turn out to be useful on a preparative scale (Gordillo *et al.*, 1962), although in both preparations shown here the principal peaks are sharp and well defined. The *S*-protein sample (Fig. 4*c*) gave a very broad and skewed peak. This was not due to overloading, since the peak shape was substantially the same for a fivefold reduction in the amount of the sample. When the same material was mixed with an equivalent of *S*-peptide to give the regenerated enzyme, RNase-*S*′ (Fig. 4*d*), again a sharp symmetric peak was obtained. This was clear evidence for aggregation of *S*-protein in the liquid of the column; the aggregation was confirmed by the sedimentation behavior reported below.

TABLE IV

ACTIVATION OF *S*-PROTEIN BY TRYPSIN

The substances were mixed in the *p*H stat cell at the indicated temperature in a volume of 10 ml. Aliquots containing about 10 μg of *S*-protein or RNase-*A* were removed at various times for assay. For *S*-protein 5 μg of *S*-peptide was added to the aliquots just prior to addition of substrate. In each case the initial activity measurement, set at 100%, was measured on aliquots removed just prior to the addition of the trypsin.

Compound (mg)	Trypsin (μg)	Temperature	Digestion Time (min)	No. of Bonds Split as Calculated from Alkali Uptake	Activity as % of Initial Value Before Addition of Trypsin	(Av. Individual Values)
S-protein						
20	10	25°	2	0.2	134	
			16	0.5	122	
			41	0.8	104	
			60	1.0	102	
3	10	2°	1	0	128	126, 130
			25	0	125	126, 123
3	25	2°	1	0	123	123, 122
			15	0	132	132, 132
3	75	2°	1	0	123	121, 124
			15	0	125	125, 124
RNase-*A*						
2	100	25°	30	0	100	97, 99, 103, 100

Chromatography of a total digest of *S*-protein on columns similar to those used for the runs in Figures 4 or 5 showed all the material eluting at or just behind the solvent front. Whether the absorption at 280 mμ or the ninhydrin color value was used as a measure, the elution was complete at an effluent volume of about 50 ml.

Chromatograms of partial digests presented quite a different picture (Fig. 5*a*). Three peaks were observed running well behind the solvent front (peak α) but ahead of the position expected for *S*-protein (peak δ). These peaks (β_1, β_2, γ) showed some activity when mixed with *S*-peptide but much less than that shown by undigested *S*-protein. Peak δ had the same specific activity and the same shape and position as *S*-protein, and when redigested it gave a pattern very similar to

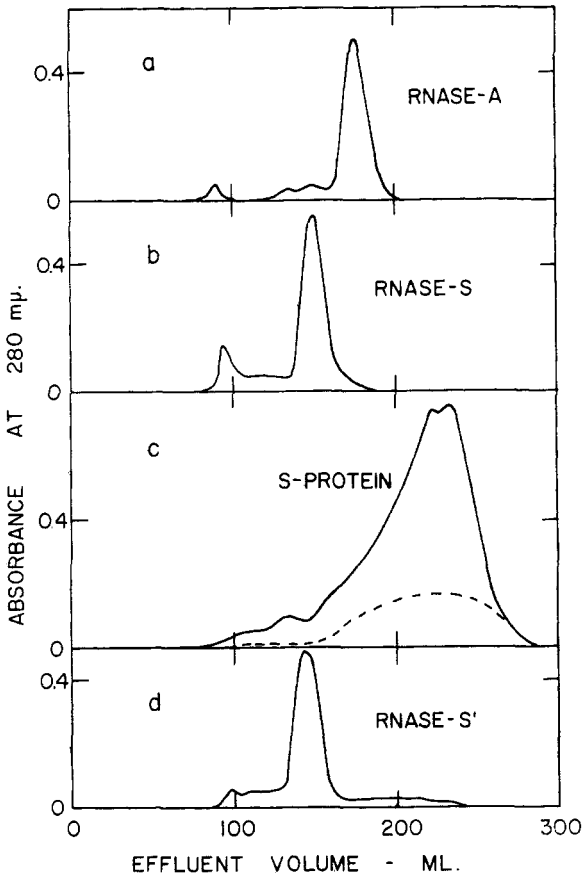

FIG. 4.—Chromatograms of various protein samples on carboxymethylcellulose. The column, 1.1 × 20 cm, was operated at 2° with gradient elution. A 1-liter closed mixing chamber was initially filled with 0.005 M Tris buffer, *p*H 8.0; the reservoir contained the same buffer made up to 0.5 M with NaCl. *a*, RNase, Armour Inc., Lot 381-059, 20 mg; *b*, RNase-*S*, 20 mg; *c*, *S*-protein, solid line 100-mg sample, dashed line 20-mg sample; *d*, RNase-*S'* made by adding 6 mg of *S*-peptide to 25 mg of the same *S*-protein sample used in part *c*.

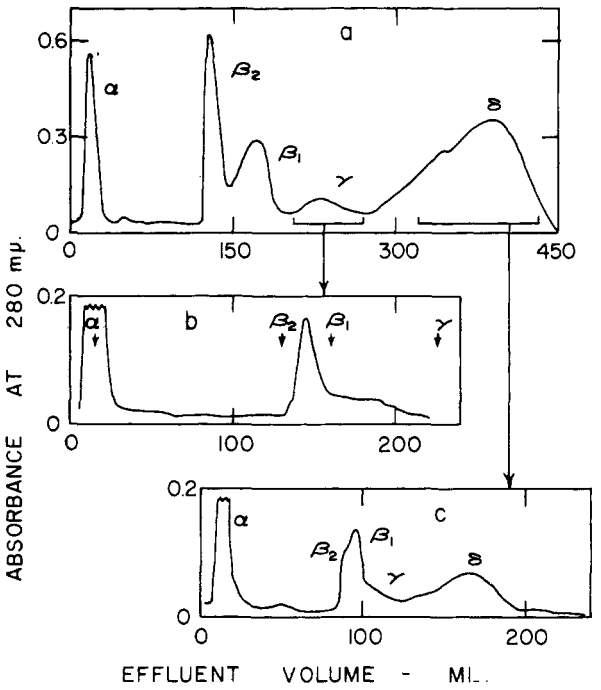

FIG. 5.—Chromatograms of partial tryptic digests of *S*-protein and some derivatives on carboxymethylcellulose. Column operation the same as in Figure 5 except as noted. *a*, 100 mg of a digest of *S*-protein having an average of one bond split and still retaining 100% activity in the presence of added *S*-peptide before chromatography. Column 1.1 × 30 cm, reservoir 0.005 M Tris buffer, *p*H 8.0, and 0.30 M NaCl. *b*, Peak γ, from a run similar to that in part *a*, was digested further with trypsin until only 20% of its activity remained. Thirty mg of this digest gave the chromatogram shown. The column was the same as in part *a*. *c*, Peak δ, from a run similar to that in part *a*, was digested further with trypsin until only 20% of its activity remained; 25 mg of this digest gave the chromatogram shown. Column 1.1 × 20 cm, reservoir 0.005 M Tris buffer, *p*H 8.0, and 0.35 M NaCl.

that of the original partial digest (Fig. 5*c*). Undoubtedly peak δ represents undigested *S*-protein in the partial digest. Peak γ on redigestion gave only fast-moving material and a peak in the region expected for β_1 and β_2 (Fig. 5*b*).

The amino acid analysis of β_2 is given in Table II. The composition agrees well with that of *S*-protein except for arginine (one residue) and possibly serine, for which the value is about 0.6 residues lower than that usually observed for *S*-protein. A seryl-arginine sequence does occur in *S*-protein and, since it is preceded by a lysyl residue (no. 31), it could be split out by the action of trypsin (Hirs *et al.*, 1960). This dipeptide may be missing from β_2. Although the agreement in the analyses of β_1 was not quite as good, it was clear that nearly the entire *S*-protein molecule was present, again with the probable exception of the seryl-arginine peptide. The amino-terminal residues reported in Table III show for both β_1 and β_2 aspartic acid or asparagine in addition to the serine present in *S*-protein. This end-group is compatible with, but not proof of, the loss of the seryl-arginine dipeptide. The tyrosine and threonine end-groups could represent the splitting of bonds 91-92 and 98–99, respectively, in the sequence of the native enzyme. In order of increasing numbers of amino-terminal residues and decreasing catalytic activity, the peaks would be listed δ, γ, β_1, β_2, α; and, although there is no proof that there is a single sequence of hydrolytic steps or that the peaks are homogeneous components, this is the probable order in which these materials are produced during the process of digestion.

The recovery of absorbance at 280 mμ on these columns was always 80 to 100%. However, the recovery of catalytic activity in the case of the partial digests was of the order of 50%. No evidence for highly active intermediates was found which might explain the peculiar activation phenomena. The lost activity was not recovered by remixing of the column fractions. Some activation of RNase-*S* was observed in the presence of excess β_1 or β_2 (see Table V), but the effect was not detectable with the amounts that could have been present

in the early stages of digestion. Peaks β_1, β_2, and γ never amounted to more than one third of the total sample according to absorbance at 280 mμ. None of these was ever more than 15% of the total. Because of their low specific activity, over 90% of the activity of a partial digest at any time was due to undigested *S*-protein.

TABLE V

ACTIVITY OF INTERMEDIATES IN THE TRYPTIC DIGESTION OF *S*-PROTEIN

Assays carried out with ribonucleic acid as substrate, measuring production of acid soluble nucleotides.

Compound	Weight Used per Assay (μg)	Molar Ratio of *S*-Peptide to Compound in Assay	Optical Density at 260 mμ over Blank (0.150)	Activity (% of that Shown by Equiv. Wt. of *S*-Protein)
S-protein	10	1.0	0.230	100
Peak β_2	50	0	0	0
		0.1	0.007	
		0.5	0.013	
		1.0	0.017	
		2.0	0.020	2
		5.0	0.021	
Peak β_1	50	0	0	0
		0.1	0.007	
		0.5	0.045	
		1.0	0.055	5
		5.0	0.060	
Peak γ	10	0	0	0
	5	2.0	0.051	
		15.0	0.047	42
		60.0	0.050	
Peak β_2 + *S*-protein	50 10	0.2 1.0	0.263	114
Peak β_1 + *S*-protein	50 10	0.2 1.0	0.265	115

Some data on the activity of these materials are given in Table V. For each of the three intermediates, very nearly maximal activity is obtained when the molar ratio of *S*-peptide to the compound in the assay is 1 or slightly greater. Strong binding between the peptide and each of the derivatives is thus indicated, although the maximum activity obtained is very much lower than that observed with equivalent amounts of *S*-protein (Richards and Vithayathil, 1960). The last two entries in Table V show that when one equivalent each of *S*-peptide and *S*-protein are mixed with 5 equivalents of either β_1 or β_2 the activity of the mixture is at least as great as that of the *S*-protein–*S*-peptide complex alone. Thus neither β_1 nor β_2 appears to be able to compete with *S*-protein for the peptide. Had they been able to, much lower activities would have been observed. The binding constant for β_1 or β_2 and *S*-peptide must be 10- to 100-fold less than that for the *S*-protein–*S*-peptide system but probably no smaller, or maximal activity would not have appeared so close to the 1:1 molar ratios discussed above. No explanation can be offered for the 15% activation noted.

Action of Trypsin on RNase-S.—The activity of RNase-*S* as a function of the number of bonds split in the presence of trypsin is shown in Figure 6. As with *S*-protein, an activation was seen in the very early stages, making any quantitative analysis of the data very difficult. In this experiment the aliquots could be assayed not only directly but also in the presence of added *S*-peptide or *S*-protein. The addition of excess peptide had no effect on the observed activity, but the addition of *S*-protein caused an easily measurable stimulation. The implication is quite clear that the first site of tryptic attack must be the *S*-protein part of RNase-*S*. In the intermediate stages of digestion there is always an excess of unhydrolyzed peptide capable of interacting with a portion of the extra *S*-protein added to the assay. Eventually both parts of the RNase-*S* molecule are completely inactivated. The data for a similar experiment with RNase-*S*′ are nearly the same as those for RNase-*S*.

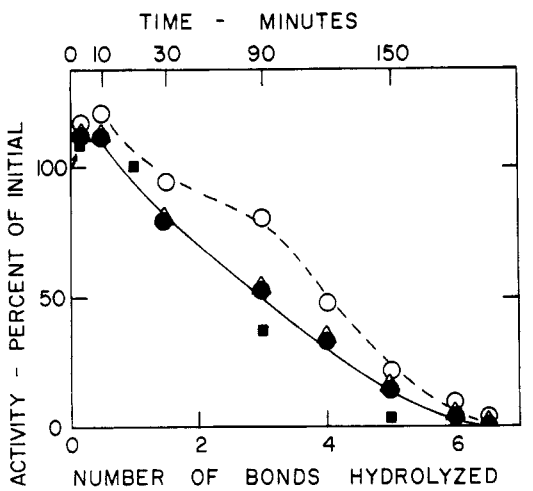

FIG. 6.—Digestion of RNase-*S* by trypsin at *p*H 8 and 25°. The sample volume was 10 ml. ■, 6.7 mg of RNase-*S*′ and 100 μg of trypsin, direct assay; ●, 7.0 mg RNase-*S* and 100 μg of trypsin, direct assay; △, same as filled circles but 7-fold molar excess of *S*-peptide added to the assay mixture; ○, same as filled circles but 3-fold molar excess of *S*-protein added to the assay mixture. The approximate time scale for the RNase-*S* digestion is shown on the top of the diagram.

Sedimentation of S-*Protein*.—The sedimentation of *S*-protein was measured in a Spinco Model E Analytical Ultracentrifuge. The dependence of the sedimentation coefficient on concentration for *S*-protein at *p*H 2.1 is shown in curve *b* of Figure 7. It has the usual negative slope found for simple protein solutions. After extrapolation to zero concentration and correction for solvent and temperature, an $s_{20,w}$ value of 1.79 *S* is obtained. This is close to the expected value for a monomer unit of molecular weight 11,500 and a shape similar to that of the native enzyme whose molecular weight is 13,700 and $s_{20,w} = 1.9$ (Harrington and Schellman 1956). The data for the same preparation made up in buffer at *p*H 8.0 are shown in curve *a* in Figure 7. The polymerization effect is clear from the marked positive slope. Even from the lowest

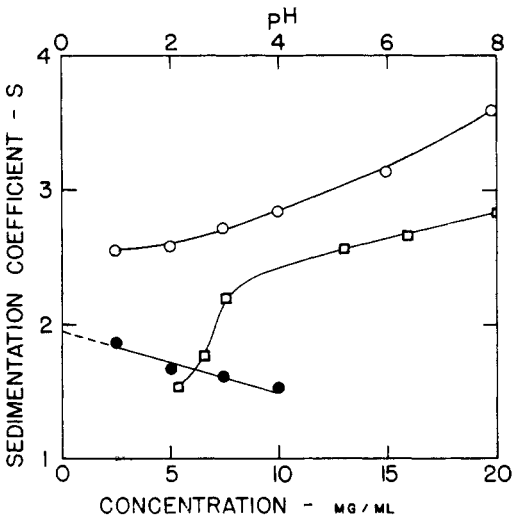

FIG. 7.—Sedimentation of *S*-protein at 24°. ○, Effect of protein concentration at *p*H 8.0, solvent 0.1 M NaCl, 0.005 M Tris adjusted with 0.1 M HCl to give *p*H 8.0; ●, effect of protein concentration at *p*H 2.1, solvent same as above except that additional HCl was added to lower the *p*H; □, effect of *p*H on 1% *S*-protein solutions, solvent same as above except for *p*H adjustment; the protein served as the buffer. The solvent used for the one point at *p*H 6.35 was 0.1 M sodium phosphate buffer. In all cases the uncorrected sedimentation coefficients are shown. These can be converted to $s_{20,w}$ values by multiplying by the factor 0.915.

concentrations measured it is not possible to extrapolate the curve to the value for the monomer. At the highest concentrations measured the sedimentation constant corresponds to aggregates larger than dimers. The patterns at all concentrations showed single slightly skewed boundaries.

The effect of *p*H on the sedimentation coefficient is shown in Figure 7, curve *c*. There is a marked change in the *p*H region 2 to 3. Changes in the structure of both *S*-protein and RNase-*S* in this same region are indicated by measurements of absorption spectra (Richards and Vithayathil, 1960). Phosphate buffer has no observable effect on the sedimentation behavior at *p*H 6.35. If the strong phosphate binding shown by RNase-*A* is present with *S*-protein, it must occur in such a way as not to influence the polymerization.

The sedimentation coefficients of a number of substances at *p*H 8 are listed in Table VI. The values for the tryptic digestion intermediates β_1, β_2, and γ are very similar to that estimated for the *S*-protein monomer under the same conditions. These results confirm the conclusions based on the amino acid analyses that each of these intermediates contains most if not all of the material present in *S*-protein.

TABLE VI

COMPARISON OF THE SEDIMENTATION COEFFICIENTS OF *S*-PROTEIN AND SOME DERIVATIVES

All measurements were made at a protein concentration of 1% in 0.1M NaCl–0.005 M Tris buffer, *p*H 8.0, as solvent. Runs were made at a controlled temperature of 24°. Measured values of *s* were multiplied by 0.915 to get $s_{20,w}$.

Compound	$s_{20,w}$
RNase-*A*	1.92
RNase-*S*	1.88
S-protein	2.59
S-protein monomer	1.41[a]
Peak β_1	1.68
Peak β_2	1.51
Peak γ	1.45

[a] The value given is for *S*-protein at *p*H 2.1 where it does appear to be a monomer. Apart from the polymerization phenomenon, neutralization to *p*H 8 would be expected to increase the $s_{20,w}$ value somewhat due to collapse of the acid expanded structure. The extent of this effect cannot be estimated from the available data.

DISCUSSION

When *S*-peptide and *S*-protein are digested separately, the total number of bonds split in *S*-peptide is 2 and in *S*-protein 7 to 8. It would be expected that the total number of bonds split in a complete digest of RNase-*S*, an equimolar mixture of the two components, would be 9 or 10. The observed value (Table I) is 6 to 7. The discrepancy is outside the limit of experimental error. The course of the proteolysis must be different in the two situations. As RNase-*S* and RNase-*S'*, the reconstituted enzyme, are identical in this regard, the difference in the course of tryptic action is clearly brought about by the association of the peptide and protein parts, and is not caused by some subtle "denaturing" effect of the initial separation procedure. Since there is no intact *S*-peptide left at the end of the proteolysis of RNase-*S* (Fig. 6), and since the fragments of *S*-peptide have been shown to interact weakly, or not at all, even with intact *S*-protein, the eventual cleavage of both susceptible bonds in the peptide portion of RNase-*S* seems fairly certain. Thus the extent of hydrolysis in the protein component is probably responsible for the discrepancy in stoichiometry. At that stage in the proteolysis where the protein and peptide components separate, the protein portion must be different in structure from an *S*-protein preparation carried to the same degree of digestion in the absence of the peptide. Were this not the case, it would be difficult to see why the subsequent course of tryptic action should then be different in these two cases. The simplest explanation would appear to be that the bonds cleaved initially in the two situations are different and that the susceptibility of the remaining bonds depends on the resulting conformational changes. These latter in turn depend upon the particular order of bond cleavage. On the other hand, if the same order of bond cleavage is assumed, different conformational changes must still be invoked and be attributed to the binding of the peptide during the early stage of digestion of RNase-*S*. In the absence of evidence to the contrary, the first explanation appears to be the most likely.

Looking at the stoichiometry from another point of view, the above evidence is a clear indication that trypsin attacks RNase-*S* as a complex and does not require dissociation into the separate

components. The kinetic data given in Figures 1, 3, and 6 provide additional support. In each case the loss of activity, or potential activity, as a function of time is more nearly first-order than zero-order and may thus be characterized, at least roughly, by a half-life. If the data are adjusted to the same trypsin concentration on the assumption that the trypsin concentration–time product is constant in any experiment, then the calculated half-lives stand in the ratio *S*-peptide: *S*-protein: RNase-*S* = 1:1.4:8.5. The rate of loss of RNase-*S* activity is thus less than 10 times slower than that of either of the separate components. Under the conditions of *p*H and temperature used for the tryptic digestion, no measurable dissociation of RNase-*S* has been observed with other techniques (Richards and Vithayathil, 1959). If trypsin acted only on the separated components, the rate of inactivation of RNase-*S* would have been much too slow to observe in the experiments described. Thus trypsin must attack the complex. It is also clear from the assays shown in Figure 6 that at least the first bond cleaved is in the *S*-protein portion of the complex. Considerable protection must be afforded to the most sensitive bond in *S*-peptide when it is bound to *S*-protein. In the separate components this appears to be the bond most rapidly split, and yet unhydrolyzed *S*-peptide, in excess of undamaged *S*-protein, can be demonstrated during the course of the digestion of RNase-*S*.

The hydrolysis of the arginyl-glutamyl bond, 10–11, in *S*-peptide is clearly responsible for the loss in activity of this component. No interaction was demonstrated between the two decapeptides so produced and *S*-protein. Because this bond was split so much more rapidly than the lysyl-phenylalanyl bond, 7–8, no appreciable amount of the tridecapeptide 8–20 was ever obtained, and thus no conclusions can be drawn about the interactions of that particular unit. The compositions of the three peptides obtained in a complete digest of *S*-peptide agree with those predicted by the sequence proposed by Hirs *et al.* (1960). However, it appears certain that residue 11 is, in fact, a glutamyl (or glutaminyl) residue as proposed by White and Anfinsen (1959) and not a seryl residue as indicated by Hirs *et al.* (1960).

Activation of RNase-*A* during digestion with carboxypeptidase was noted by Kalnitsky and Rogers (1956). The increase in activity was apparently related to the proteolysis. An explanation for the activation observed with *S*-protein and RNase-*S* remains elusive. Since maximum activation was observed before any digestion could be demonstrated, the influence of any digestion intermediates could be eliminated. Within the limits tested, the amount of trypsin used was not important nor was the observed activation effected by the addition of soybean trypsin inhibitor to the assay aliquots. Thus a very active stoichiometric complex between trypsin and *S*-protein appears unlikely. On the basis of the suggestion of Linderstrøm-Lang (1952), the activation might be due to the "denaturing" action of trypsin and not to its ability as a catalyst of hydrolysis. The reaction, thought of as a change in configuration, would have to have the following properties in order to fit the data: (1) The reaction must be very fast. Even at 2° with the smallest amount of trypsin tested it was complete in less than 1 minute. (2) The product(s) with altered configuration would have to be stable for at least 30 minutes under the conditions in the *p*H stat cell. (3) Chromatographic separation must destroy the activation, since no products with high specific activity were found and the over-all activity recoveries were low.

The chromatographic demonstration of intermediates indicates that, in the digestion of *S*-protein by trypsin, the splitting of the first bond is not the rate-limiting step. Ginsburg and Schachman (1960) have inferred the same type of behavior in the digestion of native RNase with pepsin, and they contrasted this with the action of chymotrypsin on insulin where no intermediates could be demonstrated, and thus the degradation presumably proceeded molecule by molecule.

The ultracentrifuge data and amino acid analyses indicate that all the intermediates observed in this study must have nearly the same size and shape as *S*-protein. Probably they differ only in the number of trypsin-sensitive bonds that have been hydrolyzed. The specific activities of the complexes of these materials with *S*-peptide are much less than that of RNase-*S'*, but the strength of the interactions, although also less, must still be substantial. This would indicate that the molecule maintains nearly the same configuration in spite of the cleavage of a number of bonds.

RNase-*A* has a low "helix content" (and RNase-*S* perhaps even less), as estimated by optical rotation (Schellman and Schellman, 1960) or deuterium-exchange criteria (Ottesen and Stracher, 1960). It is not difficult to picture a nonhelical, but ordered, structure with certain bonds easily accessible to proteolytic enzymes and not of prime importance in the structural integrity of the molecule. The necessary introduction of the elements of water and the probable formation of an acyl enzyme, or its equivalent, as an intermediate, make it difficult to picture the hydrolysis of a bond in an α helix without considerable disruption at least in the neighborhood of the break. One might imagine that, in substrate proteins with high helix content, the rate-limiting step might be opening up of the helix structure, and proteolysis would proceed with no demonstrable intermediates. On the other hand, substrates with low helix content would be more likely to provide isolable intermediates as in the present case. Similar conclusions have been drawn previously by others, a particularly pertinent example being the study of the tryptic digestion of myosin by Mihalyi and Harrington (1959). However, such reasoning fails to provide a satisfactory

picture of the enormous difference between the susceptibility of RNase-*A* and RNase-*S*.

The sedimentation behavior of *S*-protein in the neutral *p*H range clearly indicated polymerization, as did the chromatogram on CM-cellulose (Bethune and Kegeles, 1961). The aggregates fell apart in the presence of *S*-peptide to yield RNase-*S'*, which shows no tendency to polymerize. Qualitatively the same functional groups are present on the surface of most protein molecules. Some of these aggregate and some do not. Clearly the three-dimensional arrangement of a substantial area is important if aggregation is to occur. It is tempting to assume that *S*-protein polymerizes by making use of areas freed by the removal of the peptide. However, it is also possible that a configurational change may occur in some other part of the molecule and that this change is enough to produce an area whose geometry is suitable for polymerization. A tentative indication that the latter situation actually obtains would be the fact that none of the tryptic intermediates shows any tendency to polymerize but they do interact with *S*-peptide to give complexes with at least some enzymic activity.

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