TABLE II

THE ABILITY OF [^{14}C]ADP AND OF [^{14}C]ATP TO SERVE AS PRECURSORS

The basic incubation mixture was the same as that described in Table I, except for the omission of creatine kinase and creatine phosphate from those samples marked (—). All samples contained 23.0 A_{260} units *E. coli* s-RNA. When [^{14}C]ADP was used, 30 μmoles were added (specific activity, 0.65 μC/μmole). After incubation at 37° for 20 min, 8 ml cold 0.4 *N* $HClO_4$ were added; the precipitates were washed repeatedly, plated and counted[11].

Substrate	*Creatine kinase + Creatine phosphate*	*[^{14}C]AMP incorporated mμmole*
[^{14}C]ATP	+	0.25
[^{14}C]ATP	—	0.18
[^{14}C]ADP	+	0.23
[^{14}C]ADP	—	0.09

extracts. Detailed comparative studies of the enzymes from these two sources will be presented at a later date.

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Digestion of ribonuclease A with chymotrypsin and trypsin at high temperatures

We should like to describe some experiments which offer the possibility of locating the non-helical regions in native ribonuclease and also the portions of the polypeptide chain involved in the reversible, thermal transition[1]. These experiments were based on the hypothesis that the unfolded parts of a polypeptide chain are more susceptible than the helical portions to attack by proteolytic enzymes[2]. If the amino acid sequence is known, the positions of the enzymic cleavages may be determined. In contrast to

subtilisin, which hydrolyzes a specific alanyl–seryl bond in ribonuclease A[3], both chymotrypsin and trypsin fail to hydrolyze the native protein[4]. Recent work in this laboratory[5] on the pH-dependence of the reversible, thermal transition in ribonuclease indicates that the native molecule is only partially unfolded in the transition. The portions of the polypeptide chain involved in this limited configurational change might therefore be located if the unfolding produced weak points in the molecule, *i.e.* bonds which are susceptible to proteolytic attack only under conditions where the protein has been denatured. Previously, guanidine hydrochloride has been used to effect such a structural change permitting enzymic hydrolysis[4]; however, the complications introduced by the use of guanidine can be avoided with a knowledge of the behavior of the thermal transition[5].

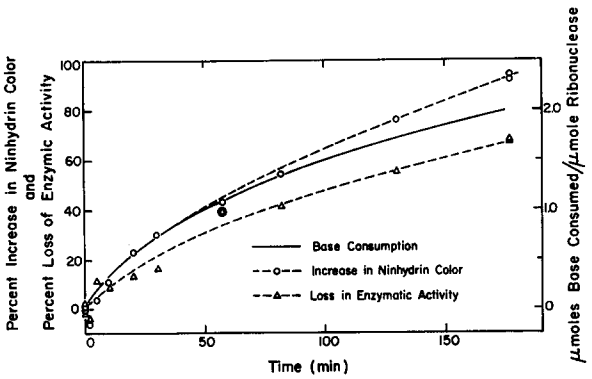

Fig. 1. The hydrolysis of ribonuclease A by chymotrypsin at pH 6.5 at 50°.

In order to locate unfolded regions of ribonuclease A, the protein was digested with chymotrypsin and trypsin at temperatures sufficiently high (40°–50°) to initiate unfolding of the molecule. Fig. 1 shows the progress of a typical hydrolysis by chymotrypsin at pH 6.5 and 50°. The reaction mixture contained 9.7 mg/ml chromatographically purified ribonuclease A, 0.011 mg/ml chymotrypsin, and 0.01 *M* $CaCl_2$. A control solution of ribonuclease A which was heated at 60° for 100 min in the absence of proteolytic enzymes showed less than a 5 % change in ninhydrin color and enzymic activity. Analogous results were obtained when trypsin was substituted for chymotrypsin. In the experiment above with chymotrypsin the rate of digestion was 250 times that obtained at pH 8.0 at 25°. In another experiment, increasing the temperature from 40.3° to 43.6° resulted in a greater than 5-fold increase in the rate of digestion by chymotrypsin. This large temperature effect indicates that a configurational change in the ribonuclease molecule is most probably responsible for the increased susceptibility to proteolytic attack.

The results of the chromatographic analysis shown in Fig. 2 demonstrate that intermediates accumulate when ribonuclease A is digested with chymotrypsin at 50°. The fraction (VI) containing enzymic activity was eluted at a volume identical with that for native ribonuclease A, as determined in control experiments. The absence of ribonuclease activity in other fractions indicates that the cleavages introduced by chymotrypsin destroy the configuration required for the biological activity of this protein. Preliminary characterization of the various components by

gel filtration using Sephadex G-50 indicates that fractions II, V, VI, and VII (Fig. 2) contain material of appreciable molecular size.

Buffers containing 0.1 to 0.5 *M* sodium phosphate or citrate reduced the rate of digestion with chymotrypsin by more than 10-fold; in contrast, 0.5 *M* KCl did not greatly alter the rate. These observations are consistent with the specific effect of phosphate and other polyvalent anions in stabilizing ribonuclease A against urea denaturation[6]. The distributions of components in the chromatographic patterns of the digests made in the presence of phosphate and citrate were significantly different from the patterns of digests made in the absence of these ions.

Work is now in progress on the characterization of the various intermediates formed by digestion of partially unfolded ribonuclease with chymotrypsin and

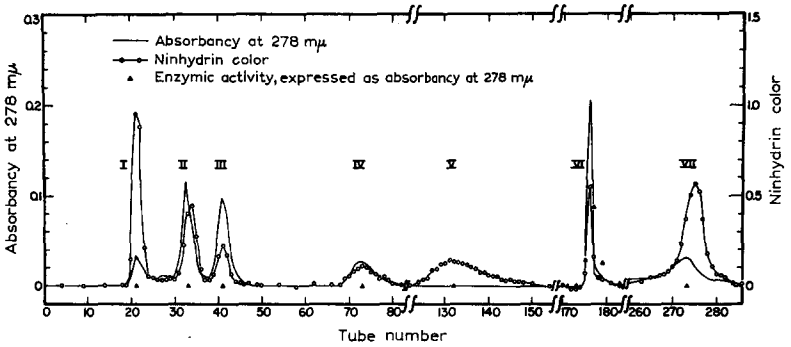

Fig. 2. The chromatographic analysis of ribonuclease A digested with chymotrypsin at pH 6.5 at 50°. The analysis was performed on a 30 × 1-cm column of IRC-50, XE-64 resin; elution was accomplished with 0.15 *M* sodium phosphate, 0.001 *M* ethylenediaminetetraacetate, pH 6.00 until tube 143, then with 1.0 *M* sodium phosphate, 0.001 *M* ethylenediaminetetraacetate buffer, pH 6.47; 0.5-ml fractions were collected and were diluted with 3.0 ml water before analysis.

trypsin, respectively. Particular emphasis will be placed on determining the sites of enzymic cleavage, and the effect of these cleavages on the physical and chemical properties of ribonuclease A. It is hoped that the positions of splits in the polypeptide chain, which occur in the early stages of the digestion, will indicate what portions of the sequence are involved in the thermal transition.

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