ARTIFICIALLY INDUCED MICROHETEROGENEITY IN RIBONUCLEASE

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Studies of the effect of temperature on the intrinsic viscosity and optical rotation of ribonuclease in water (Tanford and Weber, 1959) indicate that at pH 4.7-5.0, and an ionic strength of 0.02-0.20, the molecule starts to unfold at 50-55°, reaching a maximum at 70°. The reaction is completely reversible.

These results have been confirmed and the thermodynamic parameters ΔH and ΔS of this reversible process have been measured (Kalnitsky and Resnick, 1959).

Chromatography of crystalline bovine pancreatic ribonuclease on carboxymethyl cellulose (Taborsky, 1959) gave a marked degree of heterogeneity for a purified preparation. About two-thirds of the enzyme was found in one major peak "D", the rest being distributed among three active and one inactive peaks. The major fraction, ribonuclease "D", rechromatographed as a single component.

Since elevated temperatures and other extreme conditions are used in the preparation of ribonuclease, we decided to investigate the possibility that some of the minor ribonuclease peaks may result from these denaturing treatments. One possible result of these treatments is that slight variations in the secondary and tertiary structure of the enzyme may occur during the refolding renaturing process.

Ribonuclease "D" was prepared by the method of Taborsky with the following modifications: (a) 0.01 M borate buffer was used in place of Tris buffer, (b) a linear NaCl gradient from 0 to 0.1 M was used in the first 400 ml removed

from the column, (c) fractions of 2.0 ml were assayed for ultraviolet absorbing material at 280 mu, and for ribonuclease activity in hydrolyzing RNA at pH 5.0.

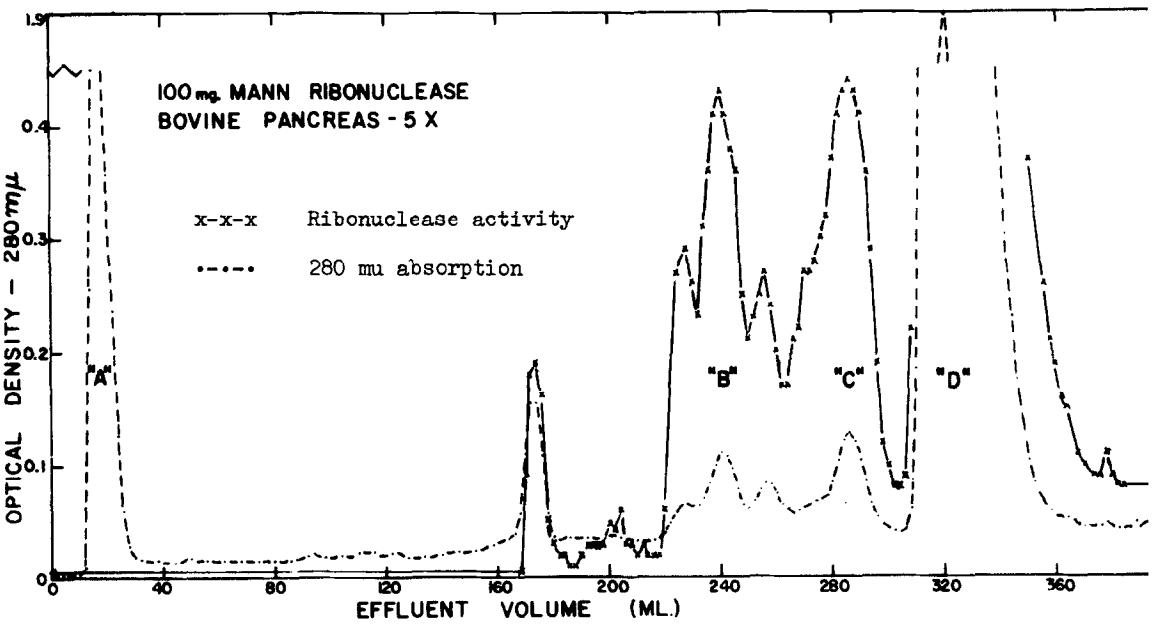

Fig. 1. Chromatography of Mann ribonuclease.

The positions of the peaks obtained compare well with those reported by Taborsky; figure 1, except when 100 mg of Mann 5x recrystallized ribonuclease was chromatographed, his "B" and "C" peaks each split into one major and two minor peaks, with indications that some of these peaks are still impure. The major portion (80%) of the "D" peak, 314-332 ml effluent volume, was used in the following experiments.

Twenty milligrams of ribonuclease "D" in 20 ml of 0.01 M borate buffer, pH 8.0 was rechromatographed and was found to be chromatographically pure in terms of 280 mu absorption and enzymatic activity. Another sample frozen for several days gave similar results.

A similar sample was heated by immersing in a boiling water bath for two minutes, then cooled rapidly to room temperature. This treatment resulted in a loss of about one-fourth of the enzymatic activity. Rechromatography of this solution; figure 2, showed a pattern similar to crystalline Mann ribonuclease except for the absence of inactive ribonuclease "A", and the occurrance

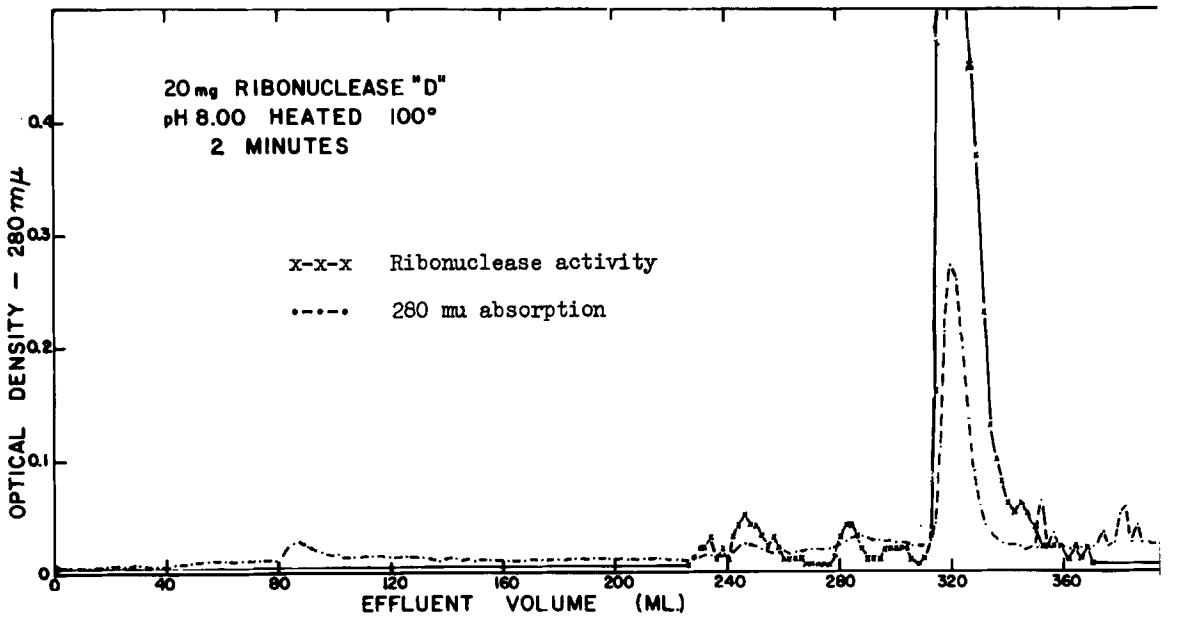

Fig. 2. Chromatography of ribonuclease "D", heated 100°, pH 8.0.

of a small inactive peak at 86 ml effluent volume. Peaks "B", "C", "D", and "E" (not shown) were present and were found to be enzymatically active. The proportion of these peaks found is similar to those found in crystalline ribonuclease.

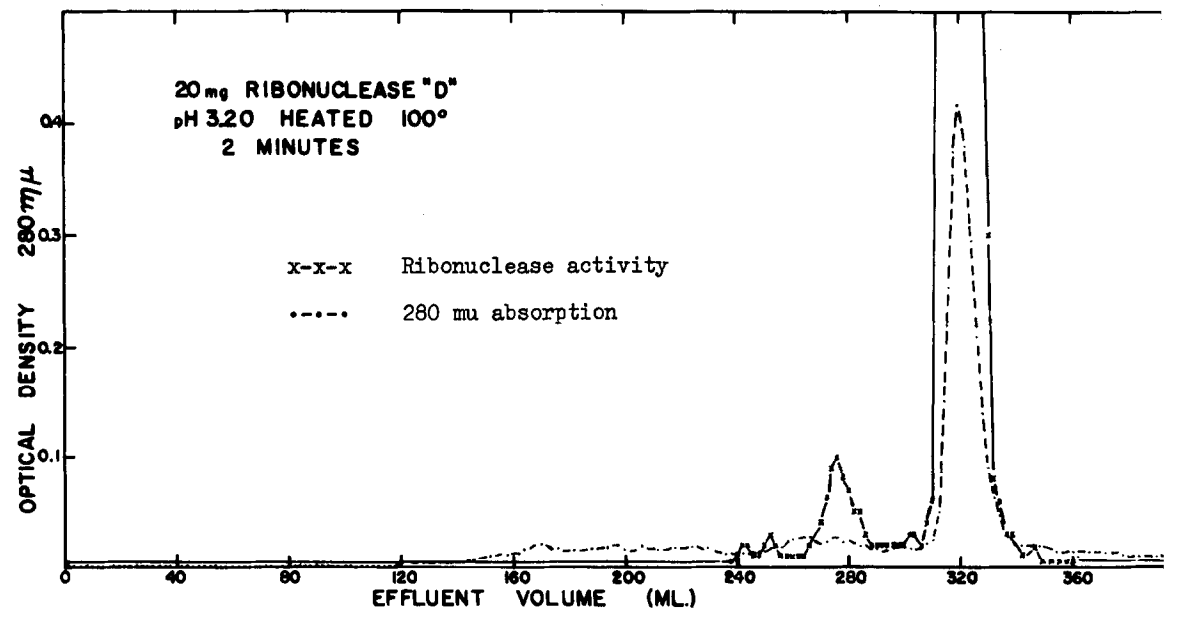

Fig. 3. Chromatography of ribonuclease "D", heated 100°, pH 3.2.

Another 20 mg sample of ribonuclease "D" was adjusted to pH 3.2 with H_2SO_4, heated as above, cooled and readjusted to pH 8.0. Almost all of the enzymatic activity was recovered. Rechromatography of this solution; figure 3, gave a pattern unlike the crystalline enzyme. Protein, as measured by the 280 mu absorption, was recovered mainly in the "D" peak, except for traces spread from 150 ml to 370 ml effluent volume, and an inactive "E" peak at 436 ml (not shown). A few rather indistinct peaks did not compare well with the major "B" and "C" peaks. Most of the enzymatic activity was found in the "D" peak, except for about 5 percent located in a minor peak just preceding the missing major "C" peak.

Other conditions of reversibly denaturing ribonuclease "D" are under investigation. These include 8 M urea, chloroethanol, extreme pH, temperature, and ionic strength. We are also investigating the effects of these conditions on the other ribonuclease fractions.

From these results, we believe that some of the active and inactive ribonucleases separated by chromatography on carboxymethyl cellulose are structural isomers, with different secondary and tertiary structures, but a similar primary structure. It is possible, of course, that terminal amino acids, peptides, or omega amino groups may have been removed during the heating procedure. We believe this to be unlikely, and are attempting to rule this out by performing end group determinations on all of the ribonuclease fractions.

At the present time, we do not know if total unfolding of the enzyme molecule is necessary in converting one kind of ribonuclease to another. This question may be answered by heating ribonuclease "D" at lower temperatures.

Each enzymatically active fraction exhibits a slightly different specific enzymatic activity (the ratio of activity to 280 mu absorption) in hydrolyzing ribonucleic acid, a result also reported by Taborsky. This finding would be expected if these fractions differed in secondary and tertiary structure. Since the enzyme has been found to be inactive when unfolded (Kalnitsky and Resnick, 1959), there is no question that the secondary and tertiary structure is essential for activity and may be responsible for the different specific

activities obtained.

Other "crystalline" enzymes have been reported to be heterogeneous on chromatography and electrophoresis. Some of this microheterogeneity may result not from differences in the primary structure of the enzyme, but from differences in the secondary and tertiary structure.

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