Studies on *B. subtilis* Ribonuclease. II. Molecular Weight and Physical Homogeneity

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The extracellular ribonuclease of *Bacillus subtilis*, in its activity and hydrolytic specificity, was found to be homogeneous with respect to molecular weight, disc electrophoresis on polyacrylamide gels, and chromatography on Sephadex G-75. The molecular weight was determined to be 10,700 ± 400 and the sedimentation coefficient $s^0_{20,w}$ = 1.5 S. The enzyme was more retarded on chromatography on Sephadex G-75 than was pancreatic ribonuclease suggesting a smaller molecular size. Amino acid analyses showed the absence of either methionine or half-cystine residues. A contaminant of molecular weight 7000, exhibiting no ribonuclease activity and separable from the enzyme by disc electrophoresis, was present in the purified preparations used. Evidence suggests that the culture medium was the source of this contaminant.

In the preceding paper (Rushizky *et al.*, 1963a) it was emphasized that the hydrolytic specificity of the extracellular ribonuclease of *Bacillus subtilis* was quite independent of the extent of its purification. It was felt, however, that more evidence was needed regarding the physical homogeneity and character of the enzyme.

Nishimura (1960) has reported that the sedimentation coefficient of *B. subtilis* ribonuclease is approximately that of bovine pancreatic ribonuclease, while preliminary measurements in this laboratory indicated that it might be significantly lower. Our value was compromised, however, by the discovery of considerable contamination with material of lower molecular size by chromatography on Sephadex G-75. Such chromatography was therefore added to our purification procedure, as described in the methods section to follow. Our results show that *B. subtilis* ribonuclease is substantially lower in molecular weight than pancreatic ribonuclease.

Materials and Methods[1]

Chromatography on Sephadex G-75 was performed at 5° with columns 366 cm long, packed in two sections in Lucite tubes. For analytical and small-scale preparative runs, the columns had an internal diameter of 0.64 cm. For larger preparative purposes, columns with an internal diameter of 2.22 cm were used. Fines were removed from the Sephadex G-75 by repeated settling and decanting, and packing of the columns was performed by gravity. During the runs flow was gravity controlled. A constant head device maintained an over-all pressure head (the vertical distance between the input and output, both being at atmospheric pressure) of 140 cm. Flow in the columns was directed upward to minimize compaction of the Sephadex. During almost continuous use over a 4-month period, the flow rate in the 0.64-cm column decreased from 3.6 ml/hour to 2.4 ml/hour, but was quite constant during any one run. Except where otherwise noted, the column was equilibrated with 0.01 M NH_4HCO_3, *p*H 8.6, and 1.0-ml samples were followed by this same buffer, which automatically regenerated the column for succeeding runs.

[1] A number of items mentioned in this paper are described in detail in the accompanying paper (Rushizky *et al.*, 1963a). Among these are the techniques of enzyme assay, the definition of the enzyme activity units, the conditions of culture of the bacteria, preliminary steps in the purification of the enzyme, and chromatography on DEAE- and CM-cellulose ion-exchangers. (Henceforth, reference to paper I is to the above-mentioned paper.)

Effluents from the column were monitored continuously with a recording ultraviolet fluorometer (G. K. Turner and Co.) and collected in tubes with a fraction collector. The fluorometer cell was a quartz tube of internal diameter 0.4 cm and length 1 cm, through which flow was directed upward. Illumination from a GE Germicidal Lamp (principally 254 mμ) was through a double-slit collimator so that only a thin horizontal disk of the flowing liquid was sampled. Fluorescent radiation was measured at right angles to the incident light through a 360-mμ filter.

For the analytical runs, extra salt was added to the sample as a marker before application to the column, and the conductivity of the effluent was recorded from a flow cell placed in series with the fluorometer. The volume of this cell was about 1 ml. It was packed with finely shredded porous polyethylene to reduce convective mixing. The emergence volume of the salt peak served as a reference point and as an added check on volume determinations from run to run. Volumes were determined by periodically marking on the recorder chart the volume remaining in the graduated cylinder which served as an input reservoir. Plotting this volume versus time always gave a straight line, from which the volume passed at any particular time could be determined. Emergence volumes were reproducible to within about 0.5 ml.

Enzyme Purification began with a culture as described in paper I, but scaled up to 300 liters and grown in a stirred fermenter with forced aeration. Cells were removed by centrifugation in a Sharples centrifuge, and acid treatment, ammonium sulfate precipitation, dialysis, and passage through DEAE-cellulose were as described in paper I for smaller batches. The material was then acidified to *p*H 4.5 and applied to a 6.0-cm × 80-cm CM-cellulose column. No unretarded enzyme corresponding to the A-peak (see paper I) appeared in this run. The column was washed with 10 liters 0.01 N sodium acetate, *p*H 4.5. As elution by a salt gradient was not practical on this scale, the enzyme was eluted sharply with 1.0 M NH_4HCO_3 and collected in about 2 liters. This was further concentrated by saturating with ammonium sulfate at 5° and dissolving the precipitate in water to a final volume of 50 ml. This was applied to the 2.2-cm × 366-cm Sephadex G-75 column. The peak of enzyme activity appeared at an effluent volume of 620 ml, and was further purified by gradient chromatography on CM-cellulose (2.2-cm × 75-cm column, see paper I) and another run on the 2.2-cm Sephadex column. The resulting preparation (no. 1) contained about 80 × 10^6 units of ribonuclease activity.

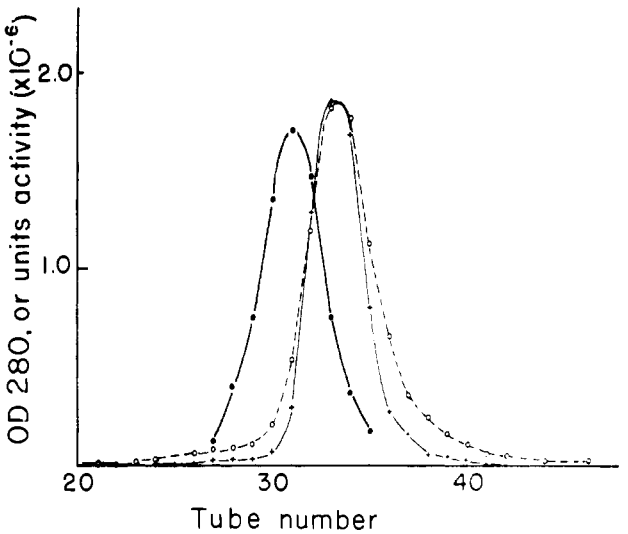

FIG. 1.—Chromatography on Sephadex G-75. Dashed line, OD_{280} of *B. subtilis* preparation no 1; light solid line ribonuclease activity in the same experiment; heavy solid line, ribonuclease activity of pancreatic ribonuclease. Column 0.64 cm × 366 cm. Solvent 0.5 N NaCl, 0.01 NH_4HCO_3, *p*H 8.6. Flow rate 2.4 ml/hour, 1-hour collections.

A portion, about 20 × 10^6 units, of preparation no. 1 was subjected to still another Sephadex run, this time on the 0.64-cm column equilibrated with 0.5 N NaCl, 0.01 N NH_4HCO_3, *p*H 8.6. The results are shown in Figure 1 in terms of OD_{280} and enzyme activity. Also shown is a curve from a comparable run on pancreatic ribonuclease. Tubes no. 33 and 34, about 9 × 10^6 units, were pooled and will be referred to as preparation no. 2.

Bovine pancreatic ribonuclease used was Type III, Lot R22B-70 from the Sigma Chemical Company. For the equilibrium sedimentation study, monomer was isolated by chromatography on the 0.64-cm Sephadex G-75 column, a sample being taken from the center of the major peak of activity.

Partial Specific Volume.—Solution density was determined by use of the density gradient column method (Linderstrøm-Lang and Lanz, 1938). The organic liquids used were *n*-dodecane and dichlorobenzene. The sample was adjusted to 0.01 N NaCl by passing it through a Sephadex G-25 column pre-equilibrated with that solvent. The small amount of salt is necessary to prevent the adsorption of this enzyme to the Sephadex.

Dry weights were determined after reaching constant weight at 110° over P_2O_5 in a vacuum oven.

Ultracentrifugation was performed with the Spinco Model E ultracentrifuge equipped with schlieren and interference optics and with the RTIC rotor temperature control. For all runs, samples were equilibrated with solvent on Sephadex G-25 columns. The solvent was 0.05 M sodium chloride, 0.02 M potassium phosphate, *p*H 7.0.

Sedimentation velocity was determined in conventional fashion, using a synthetic boundary cell and schlieren optics, from a plot of $\log_{10} X$ vs. t. Runs were performed at 50,740 rpm and near 20°. Correction to 20° in water was made in the usual fashion.

Sedimentation equilibrium was performed by the method proposed by Yphantis (1962). Interference optics are used in this technique and the fringe position corresponding to zero protein concentration is found by running at a speed high enough to maintain zero concentration in the upper part of the column. Concentration (c) may then be measured in arbitrary units as fringe displacement. Ln c is plotted against X^2 (X = distance from center of rotation) and molecular weight determined according to the equation:

$$M = \left(\frac{2RT}{(1 - \bar{\nu}\rho)\omega^2}\right)\left(\frac{d(\ln c)}{dX^2}\right)$$

where R is the gas constant, T the absolute temperature, $\bar{\nu}$ the partial specific volume, ρ the solution density, and ω the angular velocity. Assuming ideal solute behavior, a single sedimenting component will result in a straight line [slope = $d(\ln c)/dX^2$]. With a mixture, the slope at any X gives the weight average molecular weight of sedimenting components at that point in the cell.

For these experiments, 0.3 ml of protein solution was used, giving a column height of about 9 mm. The rotor was run at 50,470 rpm for at least 10 hours by which time the protein was concentrated at the bottom meniscus (FC43 fluorocarbon). The rotor was then decelerated to 35,640 rpm, the temperature being maintained at 20° and equilibrium was established in from 1 to 2 hours. Pictures for measurement were taken after 3 hours at the lower speed.

Disc electrophoresis in polyacrylamide gels at *p*H 4.5 was carried out by the method and apparatus devised for basic proteins by Reisfeld *et al.* (1962). A Spinco Analytrol densitometer was used to scan the amido-black stained gels. In order to relate enzyme activity to the pattern on the gel, 0.5-mm slices part way through the gel were taken along the gel before fixation and staining. The slices were extracted and assayed for ribonuclease activity, while the remaining continuous piece of gel was fixed and stained.

Amino acid analyses followed the method of Spackman *et al.* (1958), using the Spinco Model 120 amino acid analyzer.

RESULTS

Purification.—Figure 2 shows the results of disc electrophoresis of preparations no. 1 and no. 2. Ribonuclease activity was found only in the major peak. Pancreatic ribonuclease moved slightly faster than *B. subtilis* ribonuclease under the same conditions.

Specific activities of preparations no. 1 and no. 2 were 7.1 × 10^5 and 9.7 × 10^5 units/ml/OD_{280}, respectively, compared with 4.5 × 10^5 for our best previous preparation prepared without Sephadex chromatography. It should be emphasized, however, that these enzyme preparations, as well as a small sample taken from the major gel electrophoresis peak, did not differ from cruder preparations in hydrolytic specificity (see paper I). The activity of preparation no. 1 in units/mg was 1.05 × 10^6, compared with 1.27 × 10^6 for pancreatic ribonuclease. Yield of preparation no. 1 was 3–4% of total activity in the original culture medium.

The fast moving impurity, which imparts a yellow-brown color to the enzyme preparations, has been isolated by extraction of the heat-denatured and dried (72 hours at 110°) enzyme with water. This extract, accounting for 15–20% of the dry weight and all the color of preparation no. 1, was shown to be the fast moving contaminant on disc electrophoresis. There was negligible ribonuclease activity in this extract. However, 40% of the original enzyme activity was recovered from the residue remaining after water extraction (above) by dissolving it in 0.1 N NaOH followed by neutralization with acetic acid.

The OD_{280}/OD_{250} ratio for the impurity had the low value of 0.92; that of our best enzyme preparation (no. 2) had a value of 2.2.

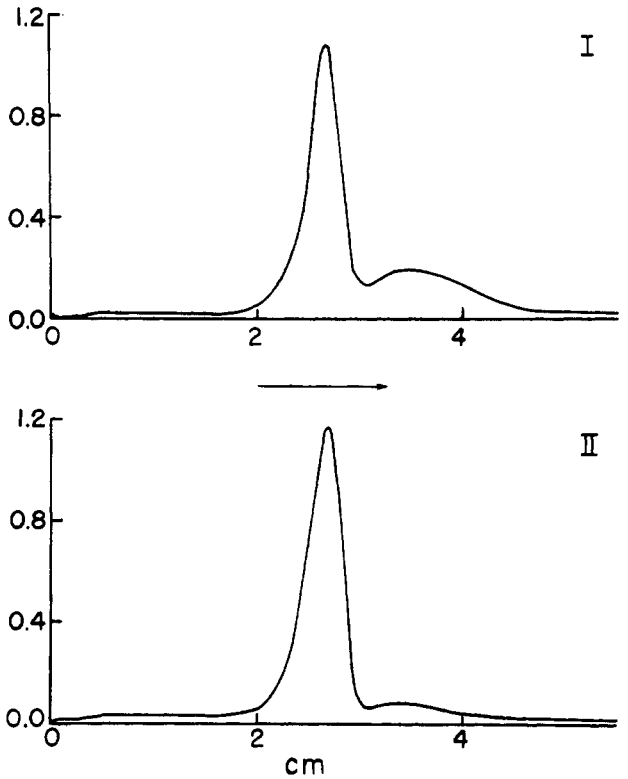

FIG. 2.—Disc electrophoresis in polyacrylamide gels. I, preparation no. 1; II, preparation no. 2, *p*H 4.5 Migration (arrow) is toward the cathode.

Analytical Chromatography on Sephadex G-75.—Emergence volumes from the 366 cm × 0.64 cm Sephadex G-75 column for *B. subtilis* ribonuclease and pancreatic ribonuclease, as well as for bovine albumin monomer, takediastase ribonuclease-T_1, and salt are given in Table I. *B. subtilis* ribonuclease and pancreatic ribonuclease, when run in the same column equilibrated with 0.5 M NaCl, 0.01 M NH_4HCO_3, *p*H 8.6, showed emergence volumes of 78 and 73 ml, respectively, indicating that the difference between the two is most likely due to a difference in molecular size and not due to differential electrostatic binding by carboxyl groups of the Sephadex. The center of the peak of enzyme activity coincided in all cases with that of the fluorescence. The width of the *B. subtilis* ribonuclease activity peak at half height was 7.7 ml, compared to 8.9 ml for pancreatic ribonuclease. This, since these widths are independent of nonenzymatic impurities, is further evidence that the activity resides in a single molecular species.

A sample of crude culture medium was also run on this column. The emergence volume of ribonuclease activity was identical to that of the purified enzyme, indicating that no gross alteration of the native enzyme had occurred during the acid treatment and subsequent purification.

TABLE I
ANALYTICAL CHROMATOGRAPHY ON SEPHADEX G-75

Sample	Emergence Volume (ml)
B. subtilis ribonuclease (prep. 1)	79.5
Bovine pancreatic ribonuclease	73.5
T_1 ribonuclease	64.5
Bovine plasma albumin	45.0
Salt	115.0 ± 1

Partial Specific Volume.—The value obtained for preparation no. 1 (80–85% enzyme) was $\bar{v}$ = 0.703. The value obtained for pancreatic ribonuclease was 0.691, which compares with the literature value of 0.695.

Sedimentation Velocity.—Preparation no. 1 was run at 50,740 rpm. Two runs were made at concentrations of about 4.5 mg/ml and 1.1 mg/ml. The $s_{20,w}$ values were 1.46 S and 1.48 S. There appears to be no significant concentration dependence under those conditions, so that $s^0_{20,w}$ = 1.5 S. Parallel runs on pancreatic ribonuclease gave $s^0_{20,w}$ = 1.9 S.

Equilibrium Sedimentation.—Figure 3 shows plots of ln *c* vs. X^2 for preparation no. 2 and for pancreatic ribonuclease monomer, both runs at 35,640 rpm as described in the methods section. The slopes of the lines drawn yield molecular weights of 10,500 and 13,500, respectively. A similar run on the impurity isolated from preparation no. 1 gave a reasonably straight line indicating a molecular weight of about 7000.

Amino acid analysis.—Preliminary amino acid analysis indicates that the enzyme has no half-cystine or methionine residues. The unexpected appearance of hydroxyproline in preparation no. 1 was fully accounted for in the isolated impurity referred to earlier. There was no hydroxyproline in the water-extracted residue from which activity was later recovered.

DISCUSSION

The major extracellular ribonuclease produced by this strain of *B. subtilis* has an exceptionally low molecular weight. Considerations of the particular small impurity with which we had to deal led us to a best value of 10,700.

While difficulties with yield and purification have forced us to use less than ideally pure enzyme solutions for our experiments, analyses of the data indicate that our major conclusion regarding the exceptionally low molecular weight is valid. To illustrate, we will assume a most unfavorable situation, e.g., a partial specific volume for the contaminant of 0.6, and 20% and 10% contamination in preparations no. 1 and no. 2, respectively. With these values, the molecular weight of the enzyme would appear to be as high as 11,900, which is still substantially lower than that of pancreatic ribonuclease. The slope of the line shown in Figure 3, furthermore, actually gives a high estimate of the weight average molecular weight, the heavier enzyme being preferentially concentrated at the bottom of the cell. The fact that the two centripetal points of this plot fall well above the line is readily accounted for by the small impurity. A more reasonable estimate of 0.7 for the partial specific volume of the impurity, even allowing the 20% and 10% contamination figures, lowers the maximum estimate of the enzyme molecular weight to 10,900, so that we feel the value of 10,700 given is probably good to within about 400.

Analytical gel exclusion chromatography also suggests a significant difference in size between *B. subtilis* ribonuclease and pancreatic ribonuclease, but the fact that the impurity of molecular weight 7000 emerges with the *B. subtilis* ribonuclease should stand as a warning. The technique is at least useful in characterizing different proteins, particularly where specific assays allow chromatography of crude preparations. It was particularly useful in our case in demonstrating that no gross changes in molecular size or configuration take place in the course of purification.

The finding that the enzyme has no half-cystine residues and hence no disulfide bridges is in agreement

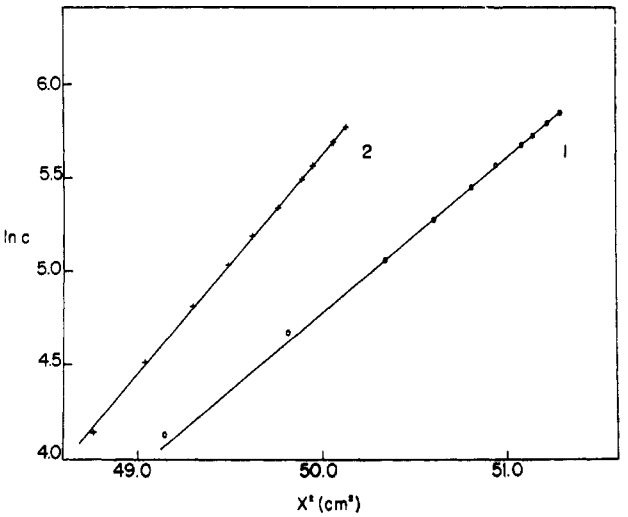

FIG. 3.—Equilibrium ultracentrifugation. 1, *B. subtilis* ribonuclease (preparation no. 2); 2, bovine pancreatic ribonuclease, *c* in arbitrary units, measured as fringe displacement, 35,640 rpm.

with the report of Nishimura and Ozawa (1962). Contrary to their report, however, we also find no methionine, suggesting that the 0.6% methionine reported in their preparation is part of a contaminant not present in ours. The value of 2.4 for the OD_{280}/OD_{250} ratio obtained from the curve published by Nishimura and Ozawa (1962) shows however that they were not troubled by our impurity and has suggested to us that our culture medium was at fault. The finding of hydroxyproline in the impurity supports this suspicion, as we had substituted the Neopeptone meat derivative for their soy bean extract. We are hopeful that changes in our culture medium will allow significant increases in both yield and purity of our preparations. Some 50% of our enzyme loss occurred during dialysis, *B. subtilis* ribonuclease being small enough to leak through the membrane. We hope to avoid this loss by means of a desalting step involving extraction of the enzyme into phenol (Rushizky *et al.*, 1963b).

The ultraviolet fluoroescence monitor has proven very useful. Its sensitivity for *B. subtilis* ribonuclease is about two orders of magnitude greater than ultraviolet absorption measurement at 280 mμ. Furthermore, the double-beam instrument used gives an extremely stable baseline and a wide range of sensitivities (achieved by adjusting the width of the illuminating slits). The output is proportional to protein concentration up to about 1 mg/ml and may be recorded with a simple 1-milliampere recorder. It is not, however, so suitable for general scanning of protein mixtures, as its sensitivity for different proteins varies considerably. *B. subtilis* ribonuclease, presumably because of its high tryptophan content, gives a response about ten times that of plasma albumin (with two tryptophans per 65,000). The response from pancreatic ribonuclease, which has no tryptophan, is lower than albumin by another factor of 10. A 303-mμ interference filter to detect tyrosine fluorescence would probably increase the sensitivity for proteins lacking tryptophan (Teale, 1960).

The low molecular weight of this enzyme, combined with its stability, the presumed simplicity of its primary structure (there being no disulfide bridges), and its high enzymatic activity makes *B. subtilis* ribonuclease an attractive target for a wide variety of experiments related to enzyme structure and function. To this may be added the fact that it is produced in relatively high yield by an organism amenable to the techniques of modern bacterial genetics.

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