Studies on *Bacillus subtilis* Ribonuclease. III. Purification and Amino Acid Composition*

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ABSTRACT: The purification of the extracellular ribonuclease from *Bacillus subtilis* has been simplified by the use of a chemically defined growth medium. An over-all recovery of 26% yielded 0.21 mg of the purified enzyme/l. of culture medium. The final preparation showed a single band on disc electrophoresis at pH 4.5. However, disc electrophoresis at pH 2.3 and the presence of 0.2 mole (per mole) of methionine suggest that the material is still heterogeneous. It is estimated that the purity of the preparation is greater than 90%. Double immunodiffusion shows a single band at pH 8. Two bands which appear at pH 6 are attributed to antibody heterogeneity.

The amino acid composition is presented and the lack of cystine is confirmed. Absence of sulfur is suggested for the pure enzyme. Alanine was the only N-terminal amino acid detected (by the 2,4-dinitrophenyl and 1-dimethylaminonaphthalene-5-sulfonyl methods) and no new N-terminal groups were detected after cyanogen bromide treatment.

Nishimura and Nomura (1958), in the course of a study of amylase production by *Bacillus subtilis*, reported that the cells also produced an extracellular ribonuclease. A later report (Nishimura, 1960) that this enzyme was specific for the secondary phosphate esters of purine riboside 3′-phosphates in ribonucleic acid (RNA), stimulated detailed chromatographic analysis of the products of enzyme action upon TMV–RNA (Whitfield and Witzel, 1963), and, in this laboratory, upon yeast RNA (Rushizky *et al.*, 1963). Both groups found that although certain preferences were shown, both pyrimidine and purine linkages were hydrolyzed, and therefore the specificity was not as sharp as originally indicated.

During the course of an investigation of the physical homogeneity of this enzyme (Hartley *et al.*, 1963), evidence was obtained that its molecular weight was significantly lower than that of pancreatic ribonuclease (10,700 as opposed to 13,700). Since it is small, stable, lacks disulfide bonds, and is amenable to genetic manipulation, a detailed study of the chemical structure, enzymatic activity, and physical chemistry of this enzyme should be of interest. In this paper a simplified scheme of preparation, amino acid composition, and N-terminal amino acid analyses are presented and estimates of homogeneity discussed.

Materials and Methods

Bacterial strains, enzyme activity units, ribonuclease assay, spectrophotometric measurements, oligonucleotide mapping, and scanning of disc electrophoresis gels have been described in previous papers from this laboratory (Rushizky *et al.*, 1963; Hartley *et al.*, 1963). All chemicals were reagent grade.

Fermentation. The small fermentor used was part of a three-fermentor drive assembly, series FS-300 made by New Brunswick Scientific Co. The 80-gal fermentor was custom made for the National Institute of Arthritis and Metabolic Diseases by Stainless Steel Products Co., St. Paul, Minn. Antifoam (Dow Corning Medical Antifoam AF Emulsion) was diluted 60 ml to 10 l. and autoclaved separately.

Chromatography. Phosphocellulose (column Chromedia P-1, Whatman floc, batch 149/150, nominally 7.5 mequiv/g) was shaken dry through a nest of 18, 45, 100, 230, and 325 mesh sieves for 45 min. The selected size of adsorbent was allowed to swell for 30 min in a solution that was 0.5 M in both NaOH and NaCl. The adsorbent was then settled out of water, discharged by washing with 0.5 M HCl, and washed three times with 95% ethanol. Before packing, the phosphocellulose was equilibrated by washing twice with ten-times concentrated starting buffer, and three times with starting buffer (potassium phosphate, pH 2.1, ionic strength 0.05). G-25 Sephadex (lot 8024C) was allowed to swell for 3 hr in water, settled from water, and equilibrated before packing by washing three times with buffer (0.005 M NH_4HCO_3, pH 8.1). CM-cellulose (Selectacel Standard, Carl Schleicher and Schuell Co., lot 1264, nominally 0.6 mequiv/g) was sieved, swelled, settled, washed, and equilibrated in the same manner as the phosphocellulose. Column effluents were monitored for A_{254} by an ISCO ultraviolet-

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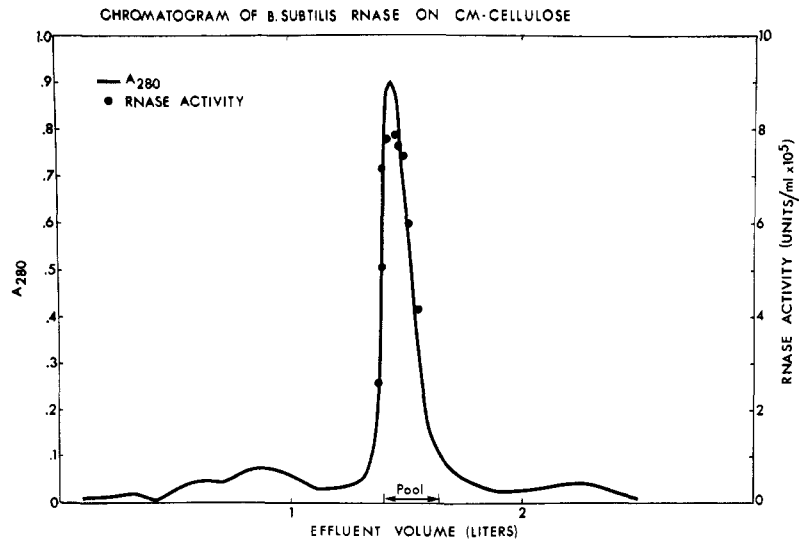

FIGURE 1: Chromatogram of *B. subtilis* ribonuclease on CM-cellulose as followed by absorbance at 280 mμ and enzyme activity. Adsorbent, Selectacel 230–325 mesh, 28 g; column, 2 × 60 cm; sample, 4, 800 ml, 140 × 10^6 enzyme units, 1100 A_{280} units; gradient, linear 0.005 M NH_4HCO_3 (pH 8.1)–0.05 M NH_4HCO_3 (pH 8.1); flow rate, 200 ml/hr; temperature, 5°.

recording densitometer (Instrumentation Specialities Co., Incorporated, Lincoln, Neb.) and for conductivity by a Radiometer conductivity meter, type CDM2, using a type CDC114 flow cell (Radiometer, Copenhagen).

Disc Electrophoresis. Disc electrophoresis following the method of Davis (1964) was carried out at pH 4.5 with the buffer system described by Reisfeld *et al.* (1962) and at pH 2.3 with a buffer system formulation described in a communication from Canalco (Canal Industrial Corp., Bethesda, Md.). Enzyme and antigen activity at positions along the gel were determined as follows. The unstained gel was frozen by placing it on a cake of Dry Ice in a paper towel and sliced into thin disks with a razor blade. (It is difficult to produce thin, symmetrical disks from an unfrozen gel.) A portion of each disk was stained with amido black. The remainder was immersed in 0.1 M Tris–chloride buffer, pH 8.5, and crushed with a stirring rod. Enzymatic and antigenic activities were measured in the supernatant after 16 hr at 5°.

Immunodiffusion. Antiserum was obtained as follows. Solid NaCl was added to an aliquot of the peak tube from the CM-cellulose fractionation (Figure 1) to make it 0.15 M in NaCl. This (3 ml) was added to 3 ml of Freund's complete adjuvant and emulsified in a Vortex mixer for 2 min. This preparation (650 μg of protein) was injected both subcutaneously and intramuscularly into a New Zealand white rabbit. The dose was repeated at 1 week. At 2 weeks 2 ml of the saline preparation was given intravenously. Bleedings of around 10 ml were performed weekly from 2 to 5 weeks at which time the animal was exsanguinated. After addition of merthiolate to make 0.01%, the serum was pooled and stored at 5°. Double immunodiffusion from circular wells was performed in a gel consisting of 1% agar (Ionagar No. 2), 0.15 M saline, and either 0.5 M glycine (pH 6) or 0.05 M barbital (pH 6–8). Plates were developed in a closed jar overnight at room temperature.

Antiserum fractionation was carried out on G-200 Sephadex (lot TO43) which was allowed to swell for 3 days in water, settled in water, equilibrated before packing by washing three times with starting buffer (0.85% NaCl–0.01 M potassium phosphate–0.01% merthiolate), pH 6.8, and packed by gravity into either a 20 × 1.1 cm (20 ml) glass column or a 182 × 0.60 cm (59 ml) Lucite column. Flow through the latter column was directed upwards. Void volumes were measured with Blue Dextran (Pharmacia).

Antiserum fractions were tested for ribonuclease inhibition by mixing aliquots with diluted enzyme and assaying after 1 hr at room temperature. Inhibition values of less than 10 or greater than 90% were repeated at appropriately lower or higher levels of enzyme. A qualitative test for precipitation of ribonuclease activity was performed by letting such a mixture stand at 5° overnight (after 1 hr at room temperature), centrifuging at 2000 rpm, 5°, for 1 hr, washing the sediment with buffered saline three times, dissolving in 0.25 M acetic acid, and assaying.

Amino Acid Composition. Acid hydrolysis was performed following the method of Moore and Stein (1963) using a Calab rotary evaporator to remove HCl. Standard runs on the amino acid analyzer (Spackman *et al.*, 1958) utilized Beckman amino acid calibration mixture, type 1, with norleucine added. Standards were run before and after the samples and each amino acid value was corrected for ninhydrin color yield, assuming a linear decrease with time. Performic acid oxidation

was carried out by the method of Moore (1963).

Tryptophan Determinations. Tryptophan assays were performed colorimetrically (Spies and Chambers, 1949) and spectrophotometrically (Bencze and Schmid, 1957). Tryptophan (General Biochemicals Inc., 98% pure by ninhydrin CO_2) was used as a primary standard for the colorimetric assay.

End-Group Determinations. Dinitrophenylation of the protein was performed by the method of Sanger (1945). Extraction of excess reagent, acid hydrolysis, and ether extraction were carried out as described by Fraenkel-Conrat *et al.* (1955). DNP-amino acids were identified by thin layer chromatography in the Eastman Chromogram Apparatus on Eastman Chromogram silica gel sheets type K 301R (with fluorescent indicator), utilizing the benzene–acetic acid–pyridine (70:6:24) and (90:2:8) solvent systems of Brenner *et al.* (1962), and the organic phase of the toluene–pyridine–2-chloroethanol–0.8 N NH_4OH (100:30:60:60) system (Biserte and Osteux, 1951). Water-soluble DNP-amino acids were chromatographed in the 1-propanol–ammonia (70:30) system of Brenner *et al.* (1962). Quantitation was obtained by cutting out the appropriate spots, eluting with 1% $NaHCO_3$ for 15 min (Fraenkel-Conrat *et al.*, 1955), and reading the absorbance at 360 mμ.

Conditions for reaction with DNS-Cl[1] were essentially those of Gray and Hartley (1963). Enzyme (1 mg) in 1.0 ml of 0.1 M $NaHCO_3$ was added to 1.0 ml of acetone containing 5 mg of DNS-Cl and incubated for 3 hr at 35°. During the incubation the initially soluble protein formed a yellow precipitate which could be washed free of salt and other reaction products with a 50% acetone–water mixture. After acid hydrolysis as with the 2,4-dinitrophenyl (DNP) derivative, the hydrolysate was taken up in 90% acetone–10% water and analyzed on Eastman silica gel sheets type K310R2 (without fluorescent indicator). Solvent systems used included toluene–2-chloroethanol–concentrated NH_4OH (200:120:10), and the organic phases of toluene–pyridine:2-chloroethanol–0.8 N NH_4OH (100:30:60:60), and toluene–acetic acid–H_2O (200:60:5).

Cyanogen Bromide Treatment. A sample of the purified preparation was subjected to the cyanogen bromide procedure according to Gross and Witkop (1961).

Results

Enzyme Purification. B. subtilis, strain H, was inoculated into 100 ml of sterile growth medium, containing per liter: 16.9 g of monosodium glutamate (1.0 M) 5.3 g of $(NH_4)_2HPO_4$ (0.04 M), 0.75 g of KCl (0.01 M), 0.1 g of $MgSO_4$–$7H_2O$ (0.0004 M), 20 g of sucrose, and 10 ml of 95% ethanol. The culture was shaken at 37° for 20 hr and used to inoculate 10 l. of medium in a small fermentor. This fermentor was run for 26 hr at 35°, stirred at 200 rpm, and with forced aeration of 1 l./min. The resulting 10-l. culture was used in turn to inoculate an 80-gal fermentor containing 300 l. of the above sterile medium. The 80-gal fermentor (under 13-psi pressure) was run at 34° and stirred at 120 rpm, with an air flow of 360 l./min. Samples were removed periodically for pH and A_{650} measurement.

Between 32 and 39°, with adequate aeration and agitation (as obtainable in the small New Brunswick fermentor), and with careful attention to pH, an enzyme level of around 1500–3000 units/ml was obtained. With inadequate aeration, growth and enzyme production stopped before this level and were accompanied by cell lysis. In the large fermentor it was necessary to maintain the stated conditions of this experiment to avoid this outcome. The relatively low buffer concentration, required for subsequent adsorption, made necessary the occasional addition of concentrated ammonia toward the end of the growth phase to maintain the pH above 6.5. It was usually not possible to control the massive foam generated, even with automatic antifoam addition.

The log-phase doubling time of the bacterium in our medium at 34° was about 1.7 hr. Maximum enzyme levels were reached in about 36 hr. At this time cooling was begun by running chilled water through the fermentor jacket with stirring and air pressure still maintained. In 2 hr the culture temperature reached 12°. Simultaneous with the start of cooling, centrifugation was initiated by passing the culture through a large Sharples centrifuge. In 4 hr centrifugation was complete, yielding about 3 kg of sediment (mostly cells) which was discarded. After cooling to 5°, the supernatant was taken to pH 2 by the slow addition of 5.4 ml/l. of concentrated sulfuric acid, and the precipitate formed was allowed to settle out at 5° for 16 hr.

The pH 2 sediment (around 500 g) was removed by centrifugation, yielding about 260 l. of clear yellow supernatant. (The 40 l. lost up to this point represent the sum of foaming, evaporation, and mechanical losses.) The pH 2 supernatant was passed by gravity, at a flow rate of 35 l./hr, through a 19 × 45 cm (13 l.) Lucite column filled only about one-third full with 520 g of P-cellulose (18–100 mesh) and previously equilibrated with 0.05 M potassium phosphate buffer, pH 2.1. This partially filled column was found convenient, since it allowed occasional vigorous manual stirring of all the adsorbent, with the liquid level at about two-thirds of the column volume and flow temporarily interrupted. This technique achieved better equilibration, because serious channeling resulted when the liquid was allowed to flow undisturbed through such a wide adsorbent bed. Essentially no enzyme activity was detected in the column effluent.

Ammonium bicarbonate buffer, 0.01 M at pH 8.1, was then passed through the column until the pH of the effluent was around 7. This usually required about 200 l. of buffer and completely removed all color. At this point the adsorbent was repacked into a 6 × 150 cm (4.3 l.) Lucite column and washed in a single step with 3 M NaCl–1 M NH_4OH, pH 10, at a flow rate of 700 ml/hr, until the enzyme activity emerged

[1] Abbreviation used: DNS, 1-dimethylaminonaphthalene-5-sulfonyl.

TABLE 1: Purification of *B. subtilis* Ribonuclease.

Sample	Vol. (l.)	Total Act. (units $\times 10^6$)[a]	% Act.	Total Absorbance (A_{280} units)	Sp Act. (units/A_{280})	Purification
36-hr culture	262	430	100	1.89×10^6	2.3×10^2	1
pH 2 supernatant	262	510	118	1.81×10^6	2.8×10^2	1.2
P-Cellulose	1.8	240	56	1.32×10^3	1.8×10^5	800
CM-Cellulose	0.2	110	26	1.16×10^2	9.5×10^5	4000

[a] Enzyme units are defined in terms of acid-soluble A_{260} released from yeast RNA. See Rushizky *et al.* (1963) for details.

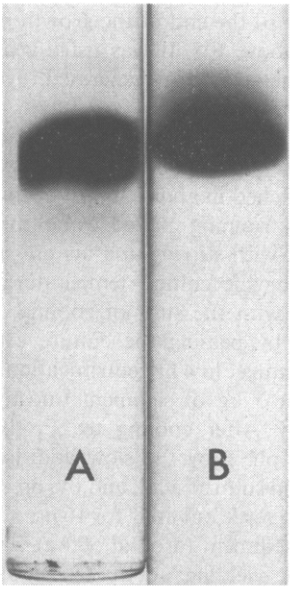

FIGURE 2: Disc electrophoresis of *B. subtilis* ribonuclease at pH 2.3 (A) and 4.5 (B). Sample, 44 μg; gel, 15% at pH 4.5 and 7.5% at pH 2.3; migration was toward cathode (descending).

in a volume of about 1 l. just ahead of the blue salt front. When the enzyme was eluted directly from the large phosphocellulose column without repacking, activity was spread out over a volume of about 40 l.

Following a buffer exchange into 0.005 M ammonium bicarbonate, pH 8.1, on a 8.3 × 60 cm (3.1 l.) column packed with 500 g of G-25 Sephadex, the material was applied to a 2 × 60 cm (170 ml) column, packed up to 15 psi with 28 g of CM-cellulose (230–325 mesh) previously equilibrated with the same buffer. The activity was eluted about half-way through a 2-l. linear gradient from starting buffer to 0.05 M ammonium bicarbonate, pH 8.1 (Figure 1). No activity was found outside the large peak, the fractions of which were pooled as indicated.

The enzyme solution, in about 0.03 M ammonium bicarbonate, pH 8.1, pooled as indicated in Figure 1, was the material used for all further studies. It represents a 26% recovery of the starting activity, a 4000-fold purification (Table I), and a yield of 0.21 mg/l. of culture medium. Its activity has remained constant within the precision of the enzyme assay, for 1 year on storage at 5° in the presence of chloroform (protein concentration 250 μg/ml).

Kjeldahl nitrogen and dry weight determinations showed 140 μg of nitrogen/787 μg of material (17.7% N). At 280 mμ, the $A^{1\%}_{1cm}$ was 20.9 in 0.03 M ammonium bicarbonate, pH 8.1, at 23°. The A_{280}/A_{250} ratio was 3.05 in 0.03 M ammonium bicarbonate–acetate, pH 5.2, at 23°. The specific activity of the final preparation was approximately 1×10^6 ribonuclease units/A_{280} unit. Paper chromatographic analysis of the oligonucleotide products of the action of this preparation upon yeast RNA indicated the same hydrolytic specificity as in earlier preparations (Rushizky *et al.*, 1963).

Tests of Homogeneity. Disc electrophoresis at pH 4.5 showed a single band at loads up to 400 μg. A retardation of the trailing edge was noted (Figure 2B) which seemed to be proportional to the amount applied. At pH 2.3, on the other hand (Figure 2A) one main band and three faint bands were seen at all sample loads. Ribonuclease activity was found only in the main band. The minor bands at pH 2.3 are more easily visualized in the gel than on the reproduction in Figure 2. Skewing of the major band at pH 2.3 was probably due to uneven photopolymerization, which ideally should be carried out using a light source which surrounds the tube. The pH 2.3 gel was scanned with a recording densitometer, and integration of the resulting peaks showed that the minor bands represent less than 10% of the total peak densities. On a similar preparation, repeated CM-cellulose chromatography, G-50 Sephadex chromatography at pH 7 and 2, CM-Sephadex chromatography, and ammonium sulfate fractionation failed to produce any further purification as judged by disc electrophoresis.

Double immunodiffusion of the purified material against pooled rabbit antiserum at pH 6 revealed two distinct precipitin bands. The main band isolated from a pH 2.3 disk also produced two immunodiffusion

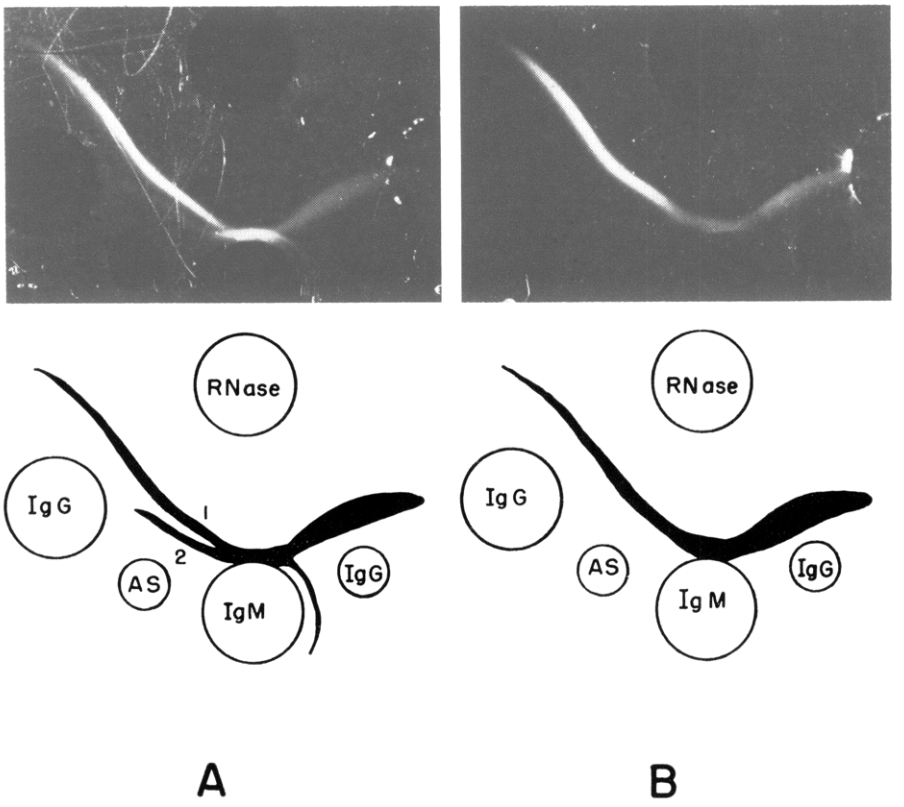

FIGURE 3: Immunodiffusion of *B. subtilis* ribonuclease. (A) pH 6, 0.15 M sodium chloride–0.5 M glycine. (B) pH 8, 0.15 M sodium chloride–0.05 M sodium barbital. AS, whole antiserum; IgM and IgG, the larger and smaller size classes of antibody as fractionated from AS on G-200 Sephadex.

bands at pH 6. After passage of 1 ml of antiserum through a small G-200 Sephadex column, the fractions were diffused at pH 6 against the purified antigen preparation. The first fraction to demonstrate antibody activity emerged just behind the totally excluded position and gave only a single band in the position of the band closer to the antiserum well (Figure 3, no. 2). The next fraction gave bands in both positions and the next three only single bands in the position farther from the antiserum well (Figure 3, no. 1). Thus, the antibody responsible for one band is of larger molecular size than that responsible for the other. We will assume that these are 19- and 7S immunoglobulins, respectively, and use the accepted abbreviations IgM and IgG. Both fractions were capable of inhibiting more than 85% of added ribonuclease activity. Ribonuclease activity was recovered from the antigen–antibody precipitates produced by both fractions.

Figure 3 shows the results of double immunodiffusion of the antigen (RNAase) against three different antibody preparations with all possible neighbor combinations of the latter. At pH 6 (Figure 3A) the IgG band joins the whole antiserum (AS) band closer to the antigen well (no. 1) in a reaction of identity. The IgM band does the same with the other AS band (no. 2), forming spurs behind the (no. 1 AS band and the IgG band from the well on the right. At pH 8 (Figure 3B) the single band formed by each antibody preparation joins with each of its neighbors, the spurs and the AS no. 2 band being absent.

In Figure 3, the pH 6 plate was buffered with 0.5 M glycine as against 0.05 M sodium barbital for the pH 8 plate. However, immunodiffusion of whole antiserum against antigen with 0.05 M sodium barbital at pH 6–8 gave two, one, and one precipitin bands, respectively.

Antiserum (1 ml) was also passed through a larger G-200 Sephadex column (see Methods) and followed by ribonuclease inhibition assay. The chromatogram (Figure 4) shows a definite, though low, level of inhibition in the nearly excluded (IgM) fractions. From this chromatogram it may be seen that the total enzyme inhibiting ability of the IgM in the whole antiserum is less than 1% of that of the IgG.

Amino Acid Composition. In Table II are listed the amino acid compositions determined for two 20-hr and two 70-hr hydrolysates, expressed as μmoles/1.05 mg of protein (equivalent to the amount applied in each column run). Asparagine and glutamine are determined as aspartic and glutamic acid, respectively. Tryptophan is destroyed. In all cases, except those noted below, the *average* value was selected as most closely reflecting the composition of the protein. The values for serine, threonine, tyrosine, and ammonia were extrapolated to zero hydrolysis time assuming

TABLE II: Amino Acid Composition of *B. subtilis* Ribonuclease.

Amino Acid	20 Hr (μmole)		70 Hr (μmole)		Av (μmole)	Nitrogen (%)	Wt (%)	Residues[d]/ Mole	Nearest Integer
Lys	1.038	1.031	0.954	0.972	0.999	15.0	12.2	10.1	10
His	0.170	0.183	0.164	0.183	0.175	3.9	2.3	1.8	2
NH_3	1.149	1.200	1.367	1.671	1.06[b]	8.0	1.8	10.8	(11)[e]
Arg	0.525	0.559	0.501	0.542	0.532	16.0	7.9	5.4	5
Asp	1.246	1.136	1.092	1.266	1.18	8.9	12.9	12.0	12
Thr	0.703	0.650	0.554	0.539	0.737[b]	5.5	7.1	7.5	8
Ser	0.653	0.591	0.417	0.387	0.740[b]	5.6	6.1	7.5	8
Glu	0.591	0.583	0.545	0.567	0.571	4.3	7.0	5.8	6
Pro	0.300	0.354	0.339	0.324	0.329	2.5	3.0	3.3	3
Gly	0.814	0.833	0.753	0.785	0.796	6.0	4.3	8.1	8
Ala	0.655	0.645	0.604	0.632	0.634	4.8	4.5	6.4	6
Cys	0	0	0	0	0	0	0	0	0
Val	0.325	0.327	0.317	0.327	0.324	2.4	3.2	3.3	3
Met	0.016	0.015	0.016	0.015	0.016	0.1	0.2	0.2	0
Ile	0.605	0.592	0.563	0.590	0.587	4.4	6.6	6.0	6
Leu	0.602	0.576	0.611	0.575	0.591	4.4	6.5	6.0	6
Tyr	—	0.493	0.472	0.600	0.502[b]	3.8	8.2	5.1	5
Phe	—	0.289	0.308	0.282	0.293	2.2	4.1	3.0	3
Trp	—	—	—	—	0.254[c]	3.8	4.5	2.6	3
Nor[a]	0.807	0.979	0.951	0.987	—	—	—	—	—
					Total	101.6	102.4	104.9	94

[a] Norleucine (1 μmole) added as internal standard. [b] Extrapolated to zero time. [c] Colorimetric and spectrophotometric determination. [d] Using 10,700 mol wt. [e] Not included in total.

first-order kinetics (Moore and Stein, 1963). The per cent nitrogen and per cent weight recoveries were 101.6 and 102.4%, respectively. Ninety-four residues per mole are calculated for molecular weight of 10,700 (Hartley *et al.*, 1963).

No cystine was detected. The presence of a small amount of methionine in this preparation was substantiated by noting that it was eluted from the resin of the amino acid analyzer in the same chromatographic position as an added methionine standard. In addition, following performic acid oxidation, this peak disappeared and two new peaks appeared in the positions of methionine sulfoxide and methionine sulfone.

The value given in Table II for tryptophan is the average of three colorimetric and three spectrophotometric determinations. The individual values were: colorimetric, 2.60, 2.60, and 2.35; spectrophotometric, 2.58, 2.54, and 2.54 (expressed as μmoles/1.05 mg of protein). The tyrosine/tryptophan ratio determined by the spectrophotometric technique was 2.0.

N-Terminal Amino Acid Determination. Duplicate 1-mg samples of the purified preparation were subjected to N-terminal amino acid analysis by the DNP method. Only DNP-alanine and ε-DNP-lysine were detected. Two quantitative DNP determinations (see Methods) on duplicate samples of 0.250 mg gave 0.91 and 0.74 mole of DNP-alanine/mole of protein after correction for losses and assuming a molecular weight of 10,700. (Recovery of DNP-alanine added as an internal standard before hydrolysis was 50%. Losses of standard DNP-alanine applied to the thin layer chromatograms were about 15%.)

Analysis of a dansylated 1-mg sample revealed only DNS-alanine, ε-DNS-lysine, and *O*-DNS-tyrosine. Sample loads ten times that necessary to produce a good alanine spot showed no other derivatives.

Discussion

The introduction of a simple, chemically defined growth medium has simplified the enzyme purification. Not only have some previous contaminants been avoided, but the early centrifugation steps have been made more efficient. The new medium will also facilitate studies, included in our long-range plans, on the incorporation of radioactive isotopes and amino acid analogs into the enzyme. The amount of enzyme in the original culture fluid is only about 25% of that obtained in the neopeptone–starch medium (Rushizky *et al.*, 1963; Hartley *et al.*, 1963). However, the recovery of the final purified preparation was 26% as contrasted with 3% obtained from the previous medium.

The large-scale growth of this bacterium for enzyme production presents certain problems. Aeration must be vigorous and continuous. Even a short period of oxygen shortage can trigger autolytic processes (Nomura,

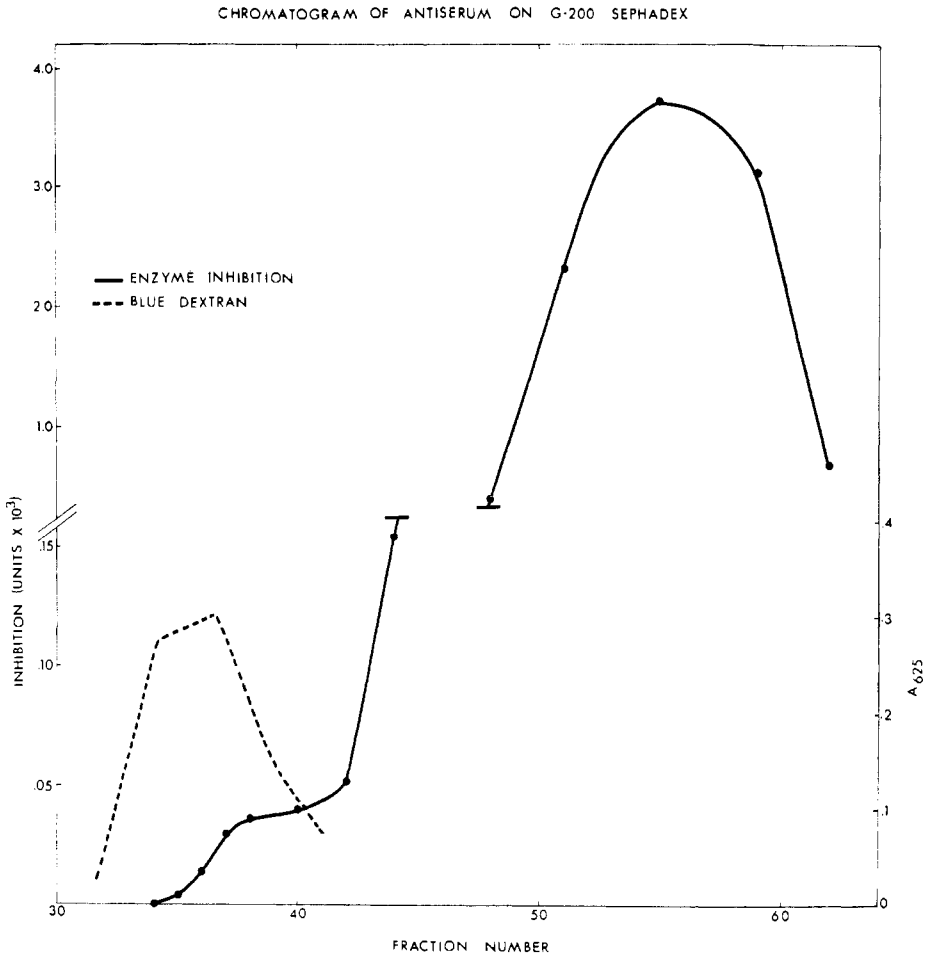

FIGURE 4: Chromatogram of 1 ml of antiserum on G-200 Sephadex as followed by inhibition of *B. subtilis* ribonuclease activity. G-200 Sephadex, 100–230 mesh, 1.5 g; column, 0.6 × 182 cm (59 ml); elution buffer; pH 6.8, 0.85% sodium chloride, 0.01 M potassium phosphate, 0.01% merthiolate; flow, 3 ml/hr, 5°. Superimposed is a chromatogram of Blue Dextran (1 ml, 0.2%) under the same conditions as monitored by absorbance at 625 mμ.

1955) with consequent reduction in enzyme yield. This reduction is presumably due largely to the release of intracellular inhibitor (Smeaton *et al.*, 1965), although proteases may also play a part. In a small fermentor, where adequate aeration is easily obtained, the bacteria may be maintained in their spent medium in stationary phase without loss of ribonuclease activity for as long as 5 days. The strong tendency to foam as the culture approaches maturity is, of course, exacerbated by the violent methods necessary for aeration. A small (200 ml/stage) two-stage, continuous-flow fermentor has been operated successfully with good yields for as long as 3 weeks. Although apparently the acid precipitation step may be carried out in the presence of the cells (Nishimura, 1960), we feel it is best to remove this rich source of contaminants as soon as possible.

An examination of the purification table (Table 1), reveals that about 50% of the enzyme activity was lost during the phosphocellulose step. This result has been confirmed in all other large-scale preparations to date, and may be due to inactivation by low salt at low pH, to irreversible adsorption, or to "tailing" from the column during elution, at a level sufficiently low to escape detection, although the last possibility seems unlikely. It is also possible that this chromatographic step removed some activating or protecting material. In any case, Nishmura (1960) also observed such a loss at a similar step.

The degree of homogeneity of the final enzyme preparation has been examined by several criteria. The CM-cellulose chromatogram (Figure 1) shows a constant specific activity throughout the main peak with the exception of the three-peak tubes, whose enzyme activities exceed the linear range of the assay. The presence of a single band on disc electrophoresis at pH 4.5 and the absence of hydroxyproline on amino acid analysis demonstrate the absence of the low molecular weight contaminant found in previous preparations (Hartley *et al.*, 1963). However, the presence of three inactive bands (in addition to the main active band) on disc electrophoresis at pH 2.3 certainly sug-

gests heterogeneity, which is estimated by densitometry at less than 10%.

If two immunodiffusion bands imply at least two antigens, as is the usual case, then the observation of two bands on immunodiffusion at pH 6, using enzyme from the major pH 2.3 disc electrophoresis band, implies heterogeneity, the degree and nature of which would be difficult to assess. Much fruitless effort, therefore, was expended in attempts to achieve further fractionation before the experiments reported here revealed an artifact. As we now interpret our results, the artifact is based on two phenomena, first, the antigen (enzyme) preparation has induced the production of serum antibodies of two size classes, presumably 7S globulins (IgG) and 19S macroglobulins (IgM). Second, the reaction between antigen and the smaller, more rapidly diffusing IgG antibody, at pH 6, but not at pH 7 or 8, is such that some antigen remains soluble in the presence of excess antibody. Thus the antigen (perhaps a soluble antigen–antibody complex) diffuses beyond the antigen–IgG precipitin band (Figure 3A, no. 1) and forms another hand (no. 2) with the larger, more slowly diffusing IgM antibody.

Crowle (1961) has described a one antigen–one antibody system giving two bands on immunodiffusion in antibody excess. This probably does not apply to our system, because we have strong evidence of at least two classes of antibody. First, the antibodies responsible for the formation of each of the two bands may be separated on Sephadex according to molecular size. Second, the larger (IgM) antibody may be selectively precipitated from whole antiserum by small amounts of antigen, implying that it differs from the smaller (IgG) antibody in antigen binding ability. This is not merely an effect of antibody dilution, because when whole antiserum is diluted to correspond to the above conditions (less than 1/5) two bands are still observed. Therefore the two bands observed at pH 6 are best explained by antibody heterogeneity, and antigen heterogeneity is not indicated. To date then, the figure obtained from disc electrophoresis at pH 2.3 (less than 10%) remains our best estimate of heterogeneity.

Several points may be made concerning the amino acid analysis. It may be seen from Table II that isoleucine and all other amino acids seem to be completely released after 20 hr of acid hydrolysis at 110°. This is in contrast to the case of pancreatic ribonuclease (Moore and Stein, 1963) and could be a reflection of the different amino acid sequence and lack of disulfide bonds in *B. subtilis* ribonuclease. It has been suggested (Tristram and Smith, 1963) that the destruction of various amino acids on acid hydrolysis may be a characteristic of the individual protein.

If the small amount of methionine detected (0.2% by weight) were a constituent of the protein at the one residue per mole level, a molecular weight of 65,000 would be implied, which is far from the values determined by ultracentrifugation, gel filtration, and end-group analysis. Methionine would account for only 0.2 residue/mole in a protein of mol wt 10,700. Therefore we consider methionine a contaminant, perhaps contributed by the minor bands seen on disc electrophoresis at pH 2.3. This implies that the pure protein would contain no sulfur. The fact that no new N-terminal residues were detected by the DNP method following treatment of the purified protein with cyanogen bromide is in agreement with the hypothesis that methionine is not present at a level of one residue per mole. An attempt to follow enzyme activity during the reaction was unsuccessful, because the reaction conditions (0.1 N HCl at room temperature for 24 hr) caused inactivation even without the addition of cyanogen bromide.

It has been of interest to examine the various presently available methods of tryptophan determination. The modification of the colorimetric method to remove interfering chloride and/or bisulfite ions (Spies, 1950) was found to be unnecessary for our samples. Both standards and sample had an absorption maximum at 590 mμ, thus eliminating the need for prior enzymatic digestion (Harrison and Hofmann, 1961). However, the fact that color developed in the sample tubes to a significant degree before the addition of oxidizing agent, suggests the presence of some oxidant in our sample preparation, which could lead to a decrease in color yield and consequent underestimation of tryptophan. The spectrophotometric method was found to be easy, rapid, sensitive, and in our hands, the method of choice. However, it is said to give difficulties when tryptophan level is low and but a small fraction of the tyrosine content (Light and Smith, 1963), as was not our case. Besides tryptophan, this method also gives the tyrosine content, which was calculated to be 0.511 μmole/sample applied to the amino acid analyzer as compared to 0.502 found by the column amino acid analysis.

The amino acid analyses are in reasonable agreement with the mol wt 10,700. Considering the precision of these analyses (Table II), it may be seen that amino acids whose residues per mole values are farthest from whole numbers (arginine, threonine, serine, alanine, and tryptophan) may be in error by one residue when rounded off to the nearest integer.

The determination of a little less than 1 mole of N-terminal alanine/10,700 g is in agreement with 10,700 as the molecular weight of *B. subtilis* ribonuclease. This, in addition to the demonstration of only alanine to be N-terminal by the DNS method, suggests that the protein consists of a single polypeptide chain. The low hydrolysis recoveries from the DNP method may reflect the presence of a relatively high amount of tryptophan in the molecule (Thompson, 1951).

The purified enzyme has the same substrate specificity and specific activity (activity/A_{280}) as that prepared in this laboratory from *B. subtilis* grown in neopeptone–starch medium (Rushizky *et al.*, 1963; Hartley *et al.*, 1963). An A_{280}/A_{250} absorbancy ratio of 3.1 as compared to that of 2.2 for the earlier preparation supports our previous conclusion that a contaminant with a low A_{280}/A_{250} ratio was being contributed by the neopeptone of the growth medium.

Our preparation may also be compared with that of Nishimura and Ozawa (1962) from the same strain

of *B. subtilis* grown in a soy bean–starch medium. Both preparations gave single peaks on CM-cellulose chromatography. They reported serine as the only N-terminal amino acid as determined by the DNP and trinitrophenyl (TNP) methods, whereas we detected only alanine by the DNP and DNS methods. The amino acid compositions may be compared in Table III. Their figures were derived from a single 24-hr

TABLE III: *B. subtilis* Ribonuclease.

Residue	Per Cent Weight: Nishimura and Ozawa (1962)	Per Cent Weight: This Paper
Lys	**7.6**	**12.2**
His	2.8	2.3
NH_3	—	(1.8)[a]
Arg	6.8	7.9
Asp	14.2	12.9
Thr	6.3	7.1
Ser	5.7	6.1
Glu	7.4	7.0
Pro	3.6	3.0
Gly	4.3	4.3
Ala	4.0	4.5
Cys	0	0
Val	3.0	3.2
Met	0.6	0.2
Ile	5.7	6.6
Leu	5.7	6.5
Tyr	8.6	8.2
Phe	6.1	4.1
Trp	5.9	4.5
Totals	98.3	100.6

[a] Not included in total.

acid hydrolysis, whereas ours are computed from averages of two 20- and two 70-hr hydrolyses. The most notable disagreement is in the lysine content. There is agreement on the absence of cystine and they too noted a small, but somewhat higher, amount of methionine. The low value, taken from their curve, of 2.4 for the A_{280}/A_{250} ratio suggests a contaminant similar to that in our earlier preparation.

The data in Table IV allow comparison of the amino acid composition of *B. subtilis* ribonuclease (as determined in this work) with that of bovine pancreatic ribonuclease and T_1 ribonuclease from *Aspergillus oryzae*. No striking homology is apparent *B. subtilis* ribonuclease has the least number of residues. Both *B. subtilis* and T_1 enzymes apparently contain no methionine but do contain tryptophan in contrast to pancreatic ribonuclease. A conspicuous feature of *B. subtilis* ribonuclease is the absence of

TABLE IV: Ribonuclease Residues per Mole.

Residue	Bovine Pancreatic[a]	*Aspergillus oryzae* (T_1)[b]	*B. subtilis*
Lys	10	1	10
His	4	3	2
NH_3	(17)[c]	(12)[c]	(11)[c]
Arg	4	1	5
Asp	15	15	12
Thr	10	6	8
Ser	16	15	8
Glu	11	9	6
Pro	4	4	3
Gly	3	12	8
Ala	12	7	6
Cys	8	4	0
Val	9	8	3
Met	4	0	0
Ile	3	2	6
Leu	2	3	6
Tyr	6	9	5
Phe	3	4	3
Trp	0	1	3
	124	104	94

[a] Smyth *et al.* (1963). [b] Takahashi (1965). [c] Not included in total.

cystine. This is most striking in the light of the important contribution of disulfide bonds to the conformation of pancreatic ribonuclease. It is tempting to postulate the presence of metal ion (by analogy with *B. subtilis* amylase, for example), to fulfill this role, although the ribonuclease activity is not affected by EDTA.

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