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Fractionation of Ribosomal Proteins by Affinity Chromatography

Corazon E. Victoria

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FRACTIONATION OF RIBOSOMAL PROTEINS
BY AFFINITY CHROMATOGRAPHY

by

Corazon E. Victoria

A Thesis
Submitted to the
Faculty of the Graduate College
in partial fulfillment
of the
Degree of Master of Arts

Western Michigan University
Kalamazoo, Michigan
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Finally, I would like to acknowledge the Department of Chemistry for the financial support and the intellectual training from its faculty which have made graduate study a pleasure and a privilege in a country that is not my own.

Corazon E. Victoria

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INTRODUCTION

One of the objectives of studies conducted in this laboratory is to explain thermophily, that is, to find out why thermophilic organisms can live at temperatures (50-80°C or higher) which, in vitro, can cause the denaturation of many macromolecules necessary for life. In comparison, mesophiles live at more moderate temperatures (20-45°C) where denaturation due to temperature is not a problem.

Three theories have been proposed to explain thermophily. The first suggests that thermophily results from the protective action of lipids (1). The second postulates a special metabolic state involving high rates of synthesis and degradation (2). The third postulates that thermophilic stability may be a result of the physical-chemical properties of important macromolecules of the organism (3). Previous work from this laboratory in support of the third theory has dealt with differences in fatty acids (4), deoxyribonucleic acid (DNA; 5), ribosomes (6), ribosomal ribonucleic acid (ribosomal RNA; 7), and ribosomal proteins (8), among others.

The ribosomes are organelles occurring in the cytoplasm of cells of animals, plants and microorganisms. They are usually attached to the outer surface of the endoplasmic reticulum, and in animal cells, also to the vesicles in the cytoplasm. Ribosomal RNA constitutes between 40 and 50%, by weight, of the ribosomes; the remainder is protein (9).

The heterogeneity of ribosomal proteins has been demonstrated (8, 10-15). The work on bacterial ribosomal proteins has long been

limited primarily to those from Escherichia coli. Research in this laboratory has shown that ribosomal proteins from the genus Bacillus are also heterogeneous (8). For those studies, three approaches to the fractionation of ribosomal proteins were investigated - countercurrent distribution, polyacrylamide gel electrophoresis, and carboxymethyl cellulose column chromatography (16). The first approach did not appear to be feasible due to experimental difficulties. The second approach yielded some fractionation but was limited to the analysis of only small amounts of ribosomal proteins. The third approach likewise resulted in some fractionation.

The present investigation was undertaken in order to develop a method for a more selective fractionation of ribosomal proteins by affinity chromatography, using a column support to which ribosomal RNA had been covalently attached.

MATERIALS AND METHODS

Chemicals

Cells:

Bacillus subtilis (high peptone medium) - General Bio-chemicals

Ribosome Isolation:

Tris (hydroxymethyl)-aminomethane (Tris) - Sigma
Magnesium acetate - Baker
Ammonium chloride - Baker
Spermidine trihydrochloride - ICN Pharmaceuticals
2-Mercaptoethanol - Eastman Kodak
Sucrose (Ribonuclease free) - Mann
Deoxyribonuclease - Worthington

Ribosomal Protein Isolation:

2-Chloroethanol - Eastman Kodak

Ribosomal Ribonucleic Acid Isolation:

Sodium dodecyl sulfate (washed in cold ethanol and ether to remove ultraviolet absorbing material) - Sigma
Phenol (redistilled immediately before use) - Matheson
Ethanol (absolute) - IMC Chemical
Sodium acetate - Mallinckrodt
Sodium chloride - Mallinckrodt

Protein Determination:

Bovine Serum Albumin (BSA) - Sigma
Sodium carbonate - Baker
Copper (II) sulfate - Fisher
Sodium potassium tartrate - Mallinckrodt
Phenol reagent - Fisher

RNA Determination:

Ferric chloride - Mallinckrodt
Hydrochloric acid - Mallinckrodt
Orcinol (recrystallized twice from benzene) - Aldrich
Ribose - Sigma
Ethanol (95%) - Mallinckrodt

Diazotization and Coupling Reactions:

m-Aminobenzyloxymethyl cellulose - Miles
Ammonium hydroxide - Fisher
Copper (II) chloride - Baker
Sodium hydroxide - Mallinckrodt
Sulfuric acid (36N) - Mallinckrodt
Sodium nitrite - Baker
Urea - Mallinckrodt
Boric acid - Sigma
Dimethylsulfoxide (DMSO) - Aldrich
Phosphoric acid - Mallinckrodt
Methylamine - Baker
2-Mercaptoethanol - Eastman Kodak

Cellulose Column Chromatography:

Cellulose phosphate (a cation exchanger;
medium mesh) - Sigma

Note: All chemicals were reagent grade.

Reagents

Ribosome Isolation:

Buffer I: 0.01 M Tris (hydroxymethyl)-aminomethane
0.01 M Magnesium acetate
0.06 M Ammonium chloride
adjusted to pH 7.4 with concentrated
Hydrochloric acid

Buffer II: Buffer I plus 0.006 M 2-Mercaptoethanol
and 0.006 M Spermidine trihydrochloride

Buffer TM/4: 0.01 M Tris (hydroxymethyl)-aminomethane
0.001 M Magnesium acetate
adjusted to pH 7.4 with
1 N Hydrochloric acid

Ribosomal Protein Isolation:

2-Chloroethanol
Rinse Solution: 1 part TM/4 Buffer
and 5 parts 2-Chloroethanol 0.06 M in
Hydrochloric acid

Ribosomal Ribonucleic Acid Isolation:

Tris Buffer: 0.01 M Tris (hydroxymethyl)-aminomethane
0.01 M Magnesium acetate,
adjusted to pH 7.4 with
1 N Hydrochloric acid

10% Sodium Dodecyl Sulfate Stock Solution
Aqueous Phenol Solution: 90% (v/v)

Acetate Buffer: 0.01 M Sodium acetate
0.10 M Sodium chloride
adjusted to pH 4.6 with
1 N Hydrochloric acid

Protein Determination:

2% Sodium carbonate in 0.1 N Sodium
hydroxide
1% Copper (II) sulfate
2% Sodium potassium tartrate
Phenol reagent, 1 N (Folin-Ciocalteu Reagent)
Bovine Serum Albumin (100 µg/ml)

RNA Determination:

0.1% Ferric chloride in concentrated HCl
 Orcinol in 95% ethanol (100 mg/ml)
 Ribose (30 µg/ml)

Diazotization and Coupling Reactions:

Copper (II) Hydroxide:

Copper (II) chloride - 25 g; dissolved in
 200 ml H₂O
 Sodium hydroxide - 15 g; dissolved in
 150 ml H₂O
 Add NaOH solution to CuCl₂ solution.
 Stir well. Vacuum filter² (Buchner) the
 sky blue precipitate of Cu(OH)₂. Wash
 at least 4 times with distilled water.
 Draw air over it to dry (overnight).
 Yield of Copper (II) hydroxide, 15 g.

Copper (II) Hydroxide Ammoniacal Solution: Per 360 ml

Copper(II) hydroxide - 16.2 g
 Sucrose - 3.6 g
 Ammonium hydroxide - 216 ml

1.8 M Hydrochloric Acid

Sodium Nitrite Solution: 10 mg per ml in water

Borate Buffer: 12.4 g Boric acid per liter,
 adjusted to pH 8 with 0.1 N NaOH

80% Dimethylsulfoxide (DMSO) Solution

Buffer 5: 0.01 M Phosphoric acid, pH adjusted to 8
 with methylamine
 0.003 M 2-Mercaptoethanol
 6 M Urea

Cellulose Column Chromatography:

Buffer 5 (0.6 M NaCl): Buffer 5, made 0.6 M in NaCl

Apparatus

French Press: Aminco, model 5-590

Homogenizer: Tri-R Instruments

Centrifuges: Sorval, model RC-2 refrigerated centrifuge
Spinco, model L preparative ultracentrifuge
International centrifuge, model K
International clinical centrifuge, model CL

Shaker: Burrell, model BB (Wrist-Action)

Spectrophotometers: Beckman, model DU-2; modified by
Update Instrument, Inc.
Bausch and Lomb, model Spectronic 20

Lyophilizer: Virtis, model 10-117

Programmed Gradient Pump: Isco, model 380 (Dialgrad)

Automatic Fraction Collector: Isco, model 326

Isolation and Purification of Ribosomes

Ribosomes from B. subtilis were isolated according to the procedure of Stenesh et. al. (17). All operations were carried out at 4°C or in crushed ice.

Approximately 300 g of cells, harvested in the late logarithmic phase, were thawed and washed with cold Buffer I. The cells were collected by centrifugation at 12,000 x g for 20 minutes and suspended with gentle stirring in 600 ml of Buffer II.

The suspension was poured into a prechilled French pressure cell. The pressure was raised to 18,000 psi in approximately one minute and maintained at that level by a slight and continuous opening of the outlet valve. The disrupted cells were released at a rate of 10 ml per minute into a container surrounded by crushed ice. Deoxyribonuclease (200 µg/25 g of cells) was added. The cell debris was removed by two 30-minute centrifugations at 30,000 x g. The first supernatant fluid was collected in its entirety. The second supernatant fluid was withdrawn to within about 1 cm above the pellet and was centrifuged at 105,000 x g for 2 hours. The resulting supernatant was discarded and the pellet was rinsed with Buffer II. The pellet represents the crude ribosomal preparation and appears as a reddish-brown gel.

The pellet was suspended in 25% the original volume of Buffer II. These crude ribosomes were further purified by a low speed centrifugation (5 minutes at 10,000 x g) and a high speed centrifugation (2 hours at 105,000 x g). The resulting pellet was suspended in 10% of the original volume of Buffer II and the solution was

centrifuged for 5 minutes at 10,000 x g to yield the final preparation of washed ribosomes which was stored at -20°C . The total volume was 64 ml.

The ribosome preparation was subsequently thawed. Two 2-ml aliquots were saved for RNA and protein determination, respectively. The remaining volume of original ribosome solution (60 ml) was divided. One half of the volume (30 ml) was used for the isolation of ribosomal proteins while the other half was used for the isolation of ribosomal RNA.

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Preparation of Ribosomal Proteins

The preparation of ribosomal proteins from ribosomes was by a modification of the procedure used by Pickett (8) and originally developed for E. coli by Fogel and Sypherd (10).

Using a prechilled glass homogenizer as a mixing vessel, five volumes of cold 2-chloroethanol were added dropwise with gentle stirring to one volume of original ribosome solution. Concentrated HCl was added dropwise with gentle stirring to a final concentration of 0.06 N. The homogenizer was placed in crushed ice and the solution homogenized at 15-minute intervals for at least 2 hours. The solution was transferred quantitatively to a centrifuge tube with Rinse Solution, the precipitated RNA was removed by centrifugation at 5,000 x g for 10 minutes, and the supernatant was carefully poured into a volumetric flask (a 25-ml flask for 3 ml of original ribosomes). The RNA pellet was resuspended in a volume of Rinse Solution equal to 30% of the original volume of ribosomes and chloroethanol. The RNA was again removed by centrifugation at 5,000 x g for 10 minutes and the supernatant was pooled with the first supernatant. The RNA pellet was rinsed with Rinse Solution which was added to the pooled supernatants. The supernatant rinsings were brought to volume with Rinse Solution. Aliquots of this solution (2 ml) were used for protein and RNA determinations in order to calculate the yields of ribosomal protein and ribosomal RNA.

It is important to remove the aliquots for assay at this point, rather than later, since ribosomal proteins are soluble in chloroethanol but are largely insoluble when the chloroethanol is

removed by dialysis.

The aliquots and the bulk of the chloroethanol solution were placed in dialysis bags and dialyzed against at least 80 volumes of distilled water to remove the chloroethanol. After 3 days of dialysis, with 6 changes of water, the bulk of the ribosomal protein was lyophilized and stored at -20°C . The dialyzed aliquots (2 ml) were transferred quantitatively to small volumetric flasks (25 ml). The proteins were dissolved by the addition of NaOH (1 mmole), the solutions were made up to volume and used for protein determination by the method of Lowry et. al. (18), and for RNA determination using the orcinol method (19). The Bausch and Lomb spectrophotometer was used for these analyses.

Isolation of Ribosomal RNA

The preparation of RNA from ribosomes was by the procedure of Stenesh and Holazo (7) which is a modification of that used by Kurland (20).

One volume of the original ribosome solution was suspended in 2.5 volumes of cold Tris buffer made 0.2% in sodium dodecyl sulfate (SDS) and incubated at room temperature for 5 minutes. An equal volume of aqueous phenol solution was added to the SDS-treated ribosomal solution and the mixture was shaken for 8 minutes at room temperature in a Burrell wrist-action shaker (setting, 5). The mixture was centrifuged for 10 minutes in a clinical centrifuge and the upper aqueous phase was pipetted from the lower phenol phase with a medicine dropper equipped with a capillary tip. The operation was repeated two more times with smaller volumes of phenol (approximately 2/3 and 1/3, respectively, of the original volume used).

The RNA was precipitated from the final aqueous phase by adding two volumes of absolute ethanol. The flocculent precipitate was brought down by centrifuging in a clinical centrifuge for 10 minutes. The supernatant containing the alcohol-soluble phenol was discarded, and the precipitate suspended in cold acetate buffer. The precipitation with absolute ethanol, centrifugation and suspension in buffer was repeated two more times. The precipitate in the last centrifugation was resuspended in cold acetate buffer and dialyzed against distilled water overnight at 4°C. The dialyzed RNA suspension was then lyophilized and stored at -20°C.

Protein Determination

Protein was determined spectrophotometrically by the Lowry modification of the Folin-Ciocalteu technique with BSA as a standard (18).

Equal volumes of 2% NaK-tartrate and 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ were combined. To one ml of this solution were added 50 ml of 2% Na_2CO_3 . Five ml of this reagent were mixed with 1.0 ml of the sample and allowed to stand at room temperature. After 10 minutes, 0.5 ml of 1 N Folin-Ciocalteu reagent was added with immediate mixing on a Vortex mixer. The absorbance was measured after 30 minutes versus a blank at either 750 nm for a 5-25 μg range or at 500 nm for a 5-100 μg range.

RNA Determination

RNA was determined spectrophotometrically using the orcinol method with ribose as a standard (19).

To the sample (3 ml) were added 3 ml of 0.1% FeCl_3 in concentrated HCl and 0.3 ml of the orcinol solution (100 mg/ml of 95% ethanol). The tubes, covered with marbles (to prevent loss due to evaporation), were heated for 40 minutes in boiling water, cooled to room temperature and the absorbance was measured at 670 and 580 nm versus a blank. The absorbance at 580 nm was subtracted from that at 670 nm to minimize glucose interference and in general to get better reproducibility.

Diazotization of Amino Cellulose and Ribonucleic Acid Coupling

The procedure was patterned after Noyes et. al. (21).

A volume of 180 ml of NH_4OH (specific gravity = 0.90) was added to 360 ml of freshly prepared $\text{Cu}(\text{OH})_2$. Undissolved material was removed by centrifugation (International centrifuge, setting at 36) for 15 minutes. The m-aminobenzyloxymethyl cellulose (3.6 g) was dissolved in this solution and the dark blue mixture was diluted with 6 volumes of water which had been heated to 70°C . The cellulose was reprecipitated by dropwise addition of concentrated H_2SO_4 to pH 2. The beaker containing the mixture was immersed in cold water during reprecipitation since otherwise the temperature rises too much. The reprecipitated cellulose was collected by centrifugation for 20 minutes and washed 3 times by resuspension in ice-cold water.

The reprecipitated cellulose was suspended in 400 ml of water at 0°C , and 800 ml of 1.8 M HCl and 320 ml of freshly prepared solution of NaNO_2 (10 mg/ml in water) were added. The reaction mixture was stirred for 60 minutes at 4°C , and excess HNO_2 was destroyed by adding solid urea until the reaction with starch-iodide paper was almost negative. The diazotized cellulose was collected by centrifugation, washed once with cold water and kept at 4°C .

Aliquots of the diazotized cellulose were washed once with cold 0.2 M borate buffer (pH 8) and once with 80% DMSO, and resuspended directly in a solution of RNA in 80% DMSO. The reaction mixture was incubated at 4°C with continual mixing (Burrell wrist-action shaker, setting at 10) for at least 48 hours. The RNA-diazotized cellulose

was washed in 80% DMSO, and at least three times in Buffer 5 before being used in column chromatography. Removal of the DMSO was followed spectrophotometrically (Beckman DU-2).

Cellulose Column Chromatography

Cellulose phosphate (7 g) was washed 3 times with distilled water, once with Buffer 5 (0.6 M NaCl), twice with distilled water and 3 times with Buffer 5. It was collected by vacuum filtration (Buchner). It was found that collecting the phosphocellulose by centrifugation (International centrifuge) resulted in finely divided particles which became unfit for column chromatography. Stirring the phosphocellulose gently and then collecting it by vacuum filtration gave satisfactory material for the column.

The cellulose was then used to prepare a 1 x 30 cm column. It was found that it was better to pack the column under mild negative pressure (vacuum; water aspirator) rather than to apply positive pressure (gradient pump) to the top of the column. When the gradient pump was first used to pack the column, there was too much compression on the upper half of the column and the run had to be terminated. In all of the subsequent experiments, the column was packed under mild negative pressure, using gentle suction.

One ml of a solution of ribosomal protein (6mg/ml) in Buffer 5 was loaded on the appropriate column and eluted with a 0-0.6 M linear gradient of NaCl in Buffer 5. The Dialgrad (Programmed Gradient Pump) was set at a flow rate of 8 ml/hr for 4 days. The elution was carried out at room temperature. Fractions (4 ml each) were collected and

analyzed for protein content.

Different proportions of RNA-diazotized cellulose were mixed with washed cellulose phosphate by gentle stirring and each mixture was used to prepare a column. The column was loaded and eluted as described previously. The 4-ml fractions were also collected and analyzed for protein content.

RESULTS AND DISCUSSION

Isolation of Ribosomal Proteins and Ribosomal RNA

The yields of ribosomal proteins and ribosomal RNA, isolated from B. subtilis, are listed in Table 1. The proteins contain 0.2% (w/w) RNA as determined by the orcinol technique. The DNA was removed by deoxyribonuclease treatment during the ribosome preparation. The proteins prepared and studied represent the proteins of the entire ribosome rather than those of a particular ribosomal subunit.

The amount of protein in the RNA preparation is estimated by the Folin reagent which can detect as little as 6 μ g protein per ml. The assay indicated that protein was absent or, at least, present in negligible amount.

Solubility of Ribosomal Proteins

The solubility of ribosomal proteins was determined using five different buffers. Solubilities were estimated by placing 1 mg of protein in a small test tube, adding 1 ml of solvent, mixing gently, and inspecting the solution visually. Solvents in which the protein is soluble become clear, those in which the protein is sparingly soluble or insoluble lead to a fine dispersion of the undissolved protein.

The following buffers were used for solubility studies:

1. 0.005 M Sodium acetate adjusted to pH 5.6 with 1 N HCl
2. 0.03 M Methylamine, adjusted to pH 5.6 with acetic acid

TABLE 1. Isolation of Ribosomal Proteins
and Ribosomal RNA from B. subtilis

Wet Weight of Cells (g)	<u>Ribosomal Protein</u>		<u>Ribosomal RNA</u>	
	Recovery (mg)	RNA Content (% w/w)	Recovery (mg)	Protein Content (% w/w)
257	541	0.20	325	0

- 6 M Urea
0.003 M 2-Mercaptoethanol
3. 0.8 ml Pyridine
4.8 ml Formic acid
1.0 ml 2-Mercaptoethanol
360 g Urea
Per liter
4. 0.05 M NaH_2PO_4
0.012 M Methylamine, pH 6.5
5. 0.01 M H_3PO_4 , pH adjusted to 8 with methylamine
0.003 M H_2PO_4^- 2-Mercaptoethanol
6 M Urea

Buffers 3 and 5 gave clear protein solutions but Buffer 3 was ruled out because of the toxicity of pyridine. Buffer 5 was used in all of the succeeding experiments.

Affinity Chromatography of Ribosomal Proteins

Affinity chromatography can be utilized to isolate some proteins from a very complex mixture. The method is based on the property of proteins for specific, noncovalent binding of another molecule, called the ligand (Figure 1). In this study, ribosomal RNA, the ligand, has been linked covalently to finely divided cellulose through a diazotized aryl amine in a method described by Noyes and Stark (21).

Figure 2 will illustrate the mechanism involved.

The method results in covalent attachment of single stranded RNA at multiple points to very finely divided cellulose; the linkage is primarily through guanine and uracil residues (21). Cavalieri and Bendich (22) and Robins (23) found that the disubstituted purine bases guanine and xanthine did couple with diazotized aromatic amines in dilute alkali at carbon 8. They also found that substitution of guanine at position 9 (as in nucleosides and nucleotides) reduces the nucleophilicity of carbon 8 so that reaction occurs instead with the primary amino substituent, or a ring nitrogen, or both (24, 25). Pyrimidine bases, uracil in RNA, probably react with diazotized aryl amines through electrophilic substitution at carbon 5 (24, 26).

Noyes and Stark (21) found that the finely divided m-amino-benzyloxymethyl cellulose has a high capacity for linking nucleic acids and that the coupling procedure is technically simple and avoids harsh treatment of the nucleic acids. However, according to them, the cellulose is not suitable for column chromatography because it is so finely divided. In order to utilize their method in the present

Figure 1. Principle of Affinity Chromatography

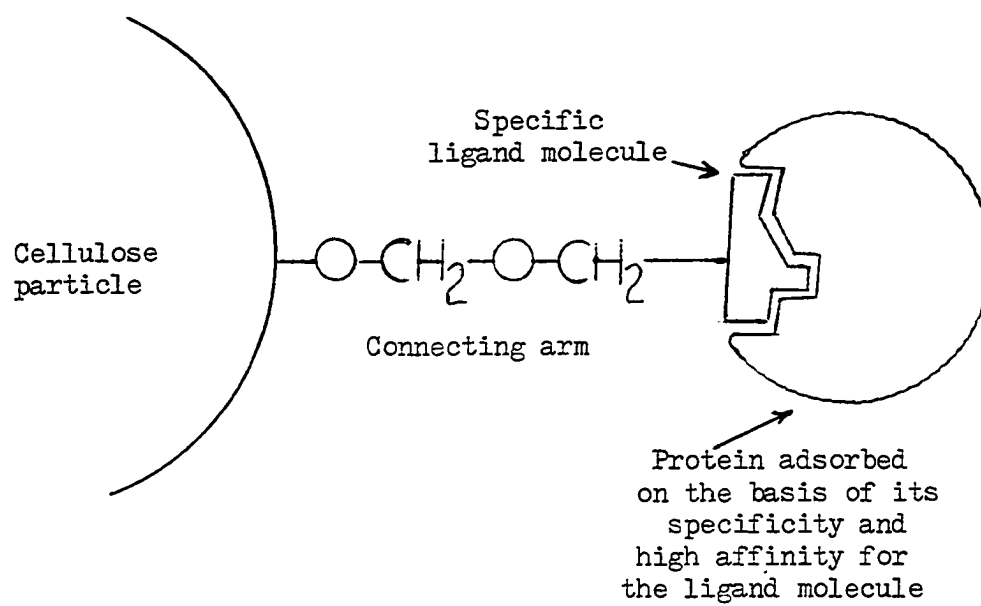
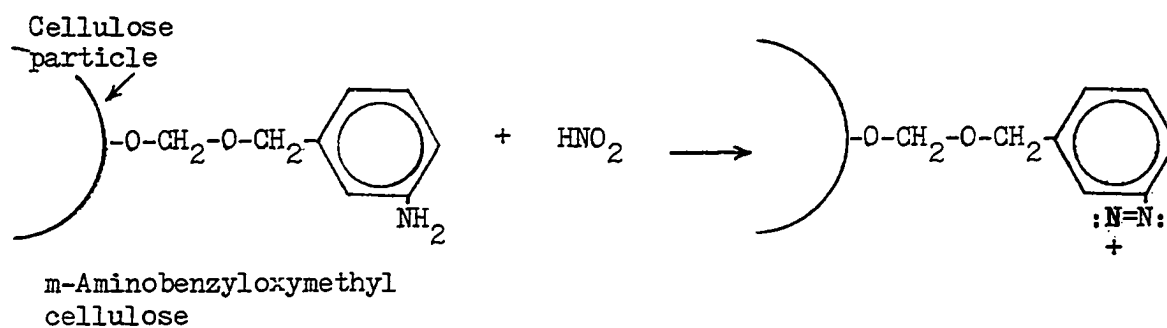
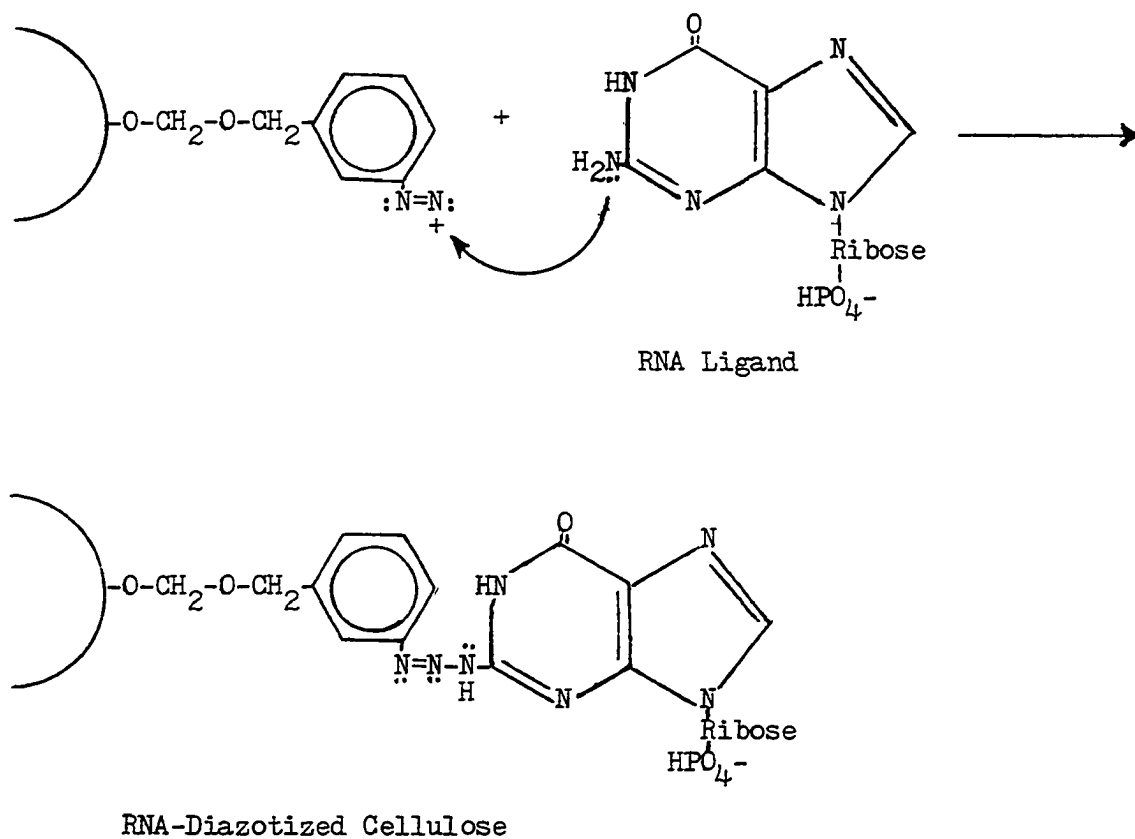


Figure 2. Fractionation of Ribosomal Proteins
by Affinity Chromatography

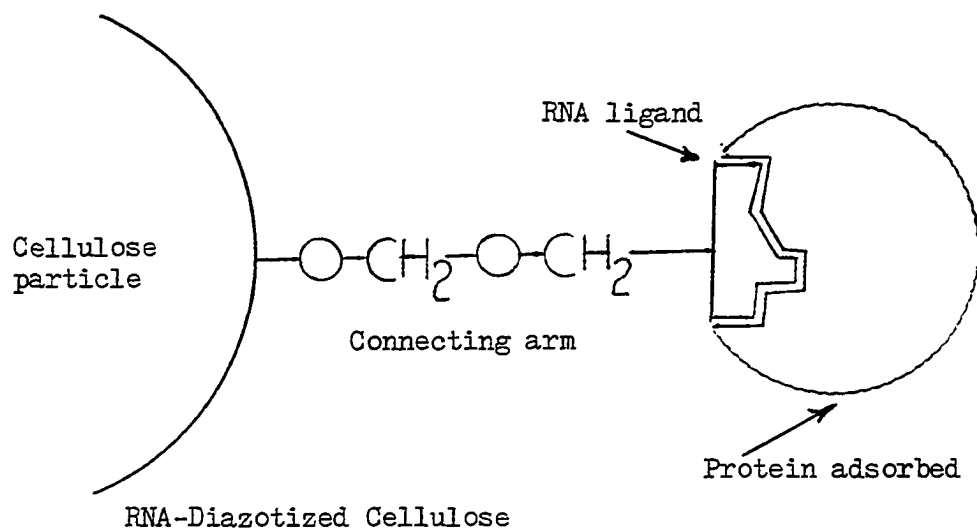
Step 1: Diazotization of m-Aminobenzylloxymethyl cellulose



Step 2: Coupling of diazotized cellulose with RNA through electrophilic substitution
(For example, with the base Guanine)



Step 3: Binding of ribosomal protein to RNA-Diazotized Cellulose



Step 4: Elution with buffer to obtain fractions of specific proteins

study, the finely divided cellulose was mixed with phosphocellulose (medium mesh) and the mixture was then used for column chromatography.

In order to be able to prepare reproducible mixtures of diazotized cellulose and phosphocellulose, the following experiments were performed: Three batches of 3.6 g m-aminobenzyloxymethyl cellulose were diazotized and the diazotized cellulose was dried to constant weight in a vacuum dessicator. The average dry weight of the diazotized cellulose was about 2.0 g.

In another experiment, an identical amount of m-aminobenzyloxymethyl cellulose (3.6 g) were diazotized, washed with water and then collected by centrifugation at 10,000 x g for 30 minutes. The weight of the wet diazotized cellulose was 46 g. It follows that 23 g of wet diazotized cellulose are equivalent to 1 g of dry diazotized cellulose.

In order to set up the 1 x 30 cm chromatographic column, 23 g of wet diazotized cellulose were required. The wet diazotized cellulose was coupled with 0.85 ml of ribosomal RNA (8mg/ml) in 80% DMSO. The RNA-diazotized cellulose was then washed with 80% DMSO and Buffer 5. The solution which appeared milky due to the very fine particles of the diazotized cellulose was loaded onto the column. There was too much compression in the column and 30 minutes after the elution began, the Tygon-tubing connectors snapped off. The run had to be stopped. A fractionation using 100% of diazotized cellulose-RNA was, therefore, not feasible. Accordingly, mixtures of RNA-diazotized cellulose and cellulose phosphate were tried. These are designated as 5, 10, and 50% diazotized cellulose-RNA by reference to the 100%

material. In other words, the 5, 10, and 50% diazotized cellulose-RNA columns contained 0.050, 0.100 and 0.500 g of dry diazotized cellulose-RNA, respectively, per column. The remainder of the volume was made up with cellulose phosphate (5.7, 4.4, and 1.0 g, respectively). The columns were prepared using the equivalent amounts of wet diazotized cellulose rather than the dry material because it turned out to be very difficult to rehydrate a previously dried diazotized cellulose. In all cases, one ml of ribosomal protein solution (6mg/ml) was applied to the column.

In order to prepare the 50% diazotized cellulose-RNA, 11.5 g of wet diazotized cellulose (500 mg dry diazotized cellulose) were coupled with 0.85 ml of RNA (8mg/ml) in 80% DMSO and then mixed with about 1 g of the washed phosphocellulose, the filler. The mixture was used to prepare the column. It became evident that this column too could not be utilized. Again, excessive compression reduced the pore openings, which in turn reduced the flow rate. Eventually, the run had to be stopped because of leakage at the tube connections.

It was decided to try lower levels of diazotized cellulose. The 5% diazotized cellulose-RNA was prepared by coupling 1.3 g of wet diazotized cellulose (50 mg dry diazotized cellulose) with 0.85 ml of RNA (8 mg/ml). Washed phosphocellulose (5.7 g) was mixed with the RNA-diazotized cellulose and loaded onto the column.

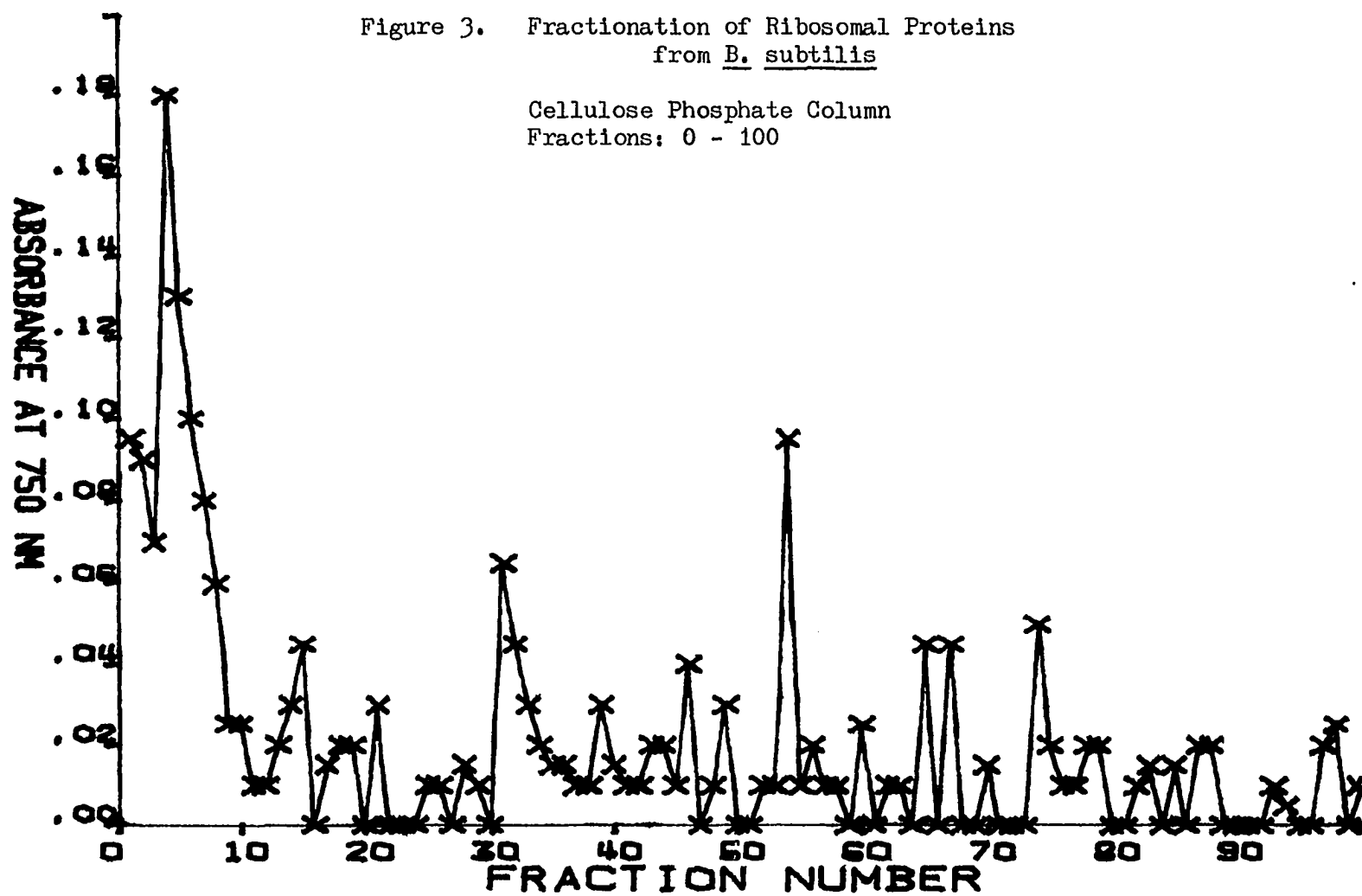
To prepare the 10% diazotized cellulose-RNA, 2.6 g of wet diazotized cellulose (100 mg dry diazotized cellulose) were coupled with 0.85 ml of RNA (8 mg/ml) and then mixed with 4.4 g of washed phosphocellulose and loaded onto the column.

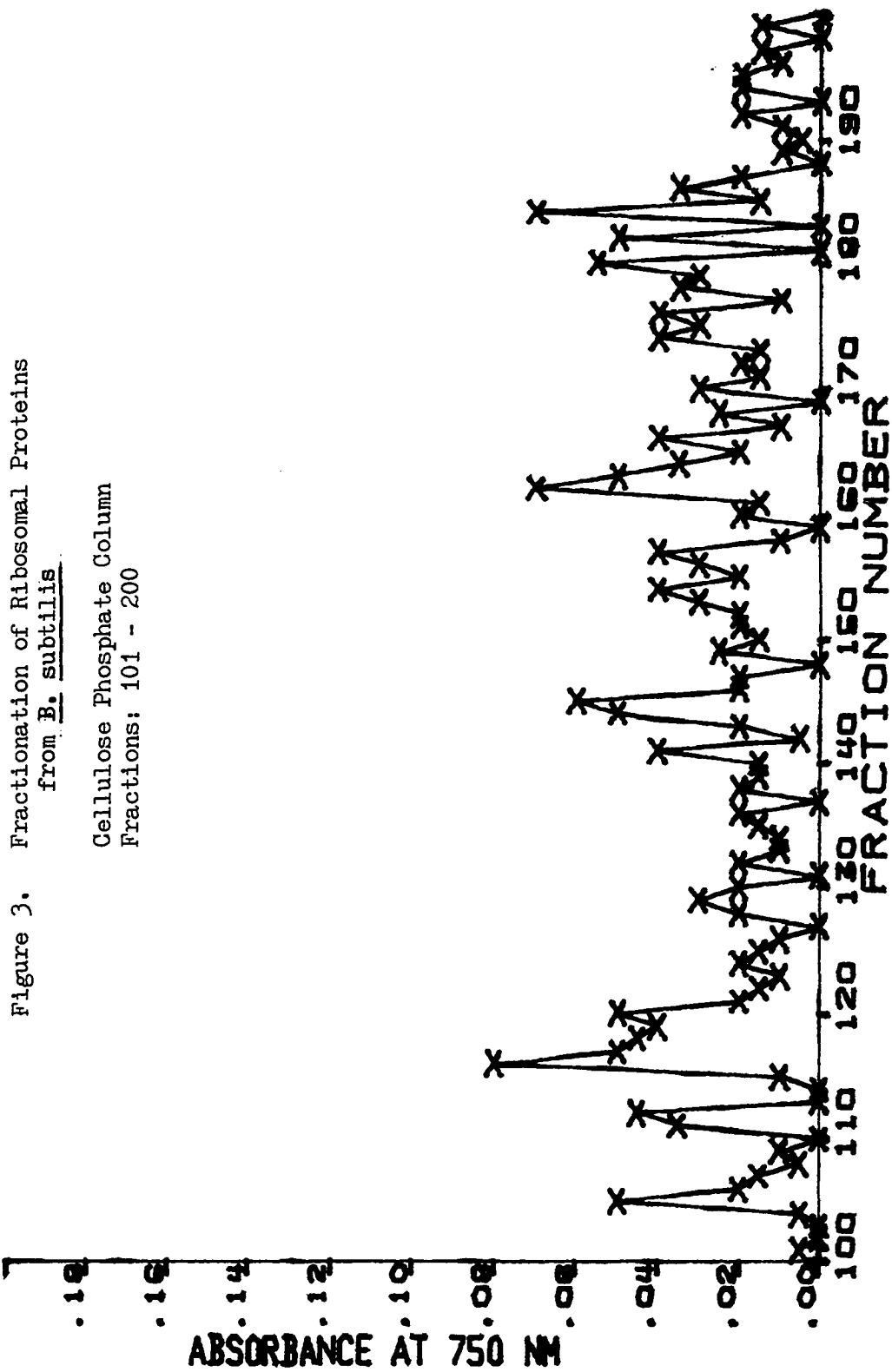
Figure 3 shows a typical elution pattern of ribosomal protein (6 mg/ml) loaded onto a column consisting only of phosphocellulose.

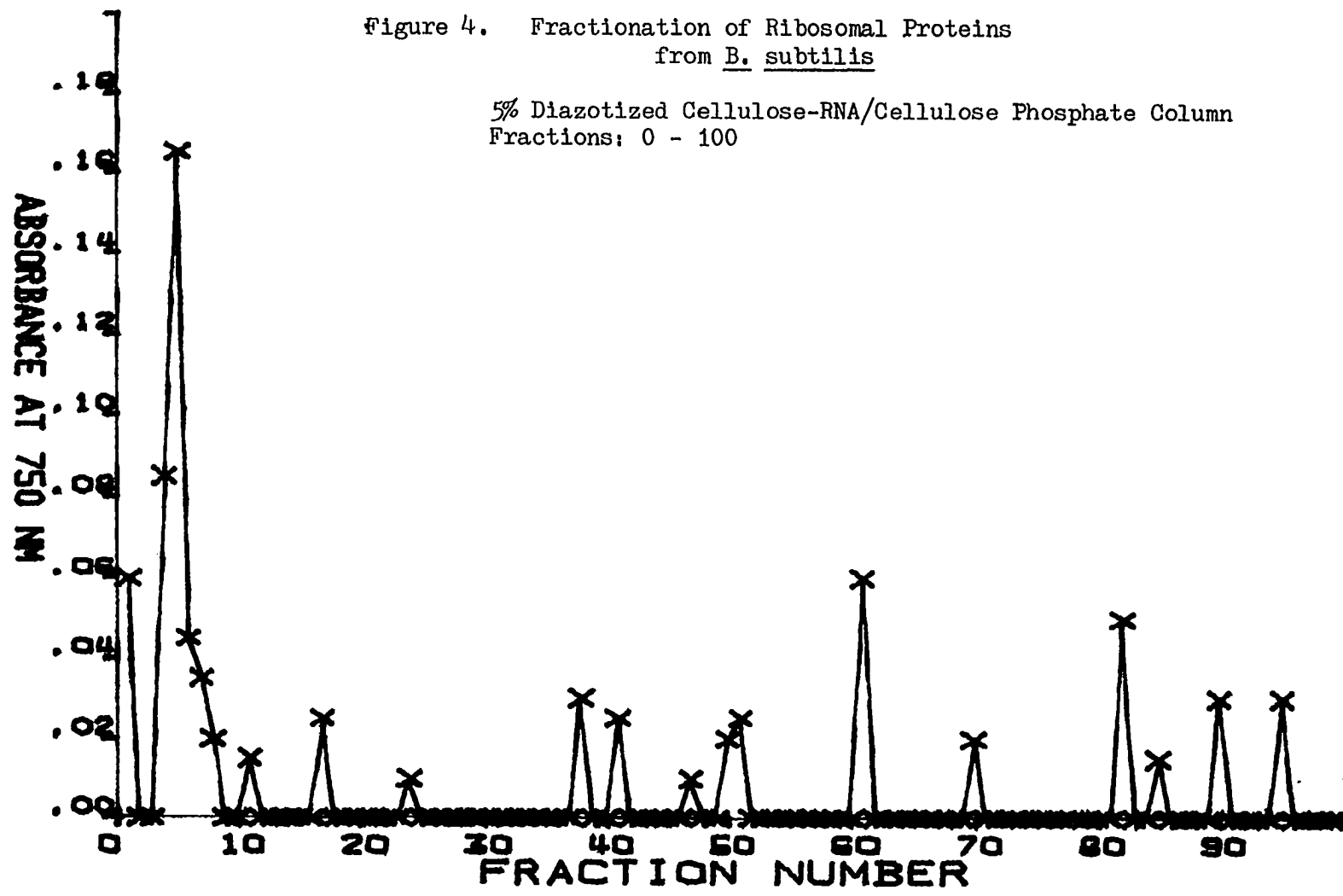
Figures 4 and 5 show the results of the complete elution series using 5% and 10% diazotized cellulose-RNA/phosphocellulose, respectively.

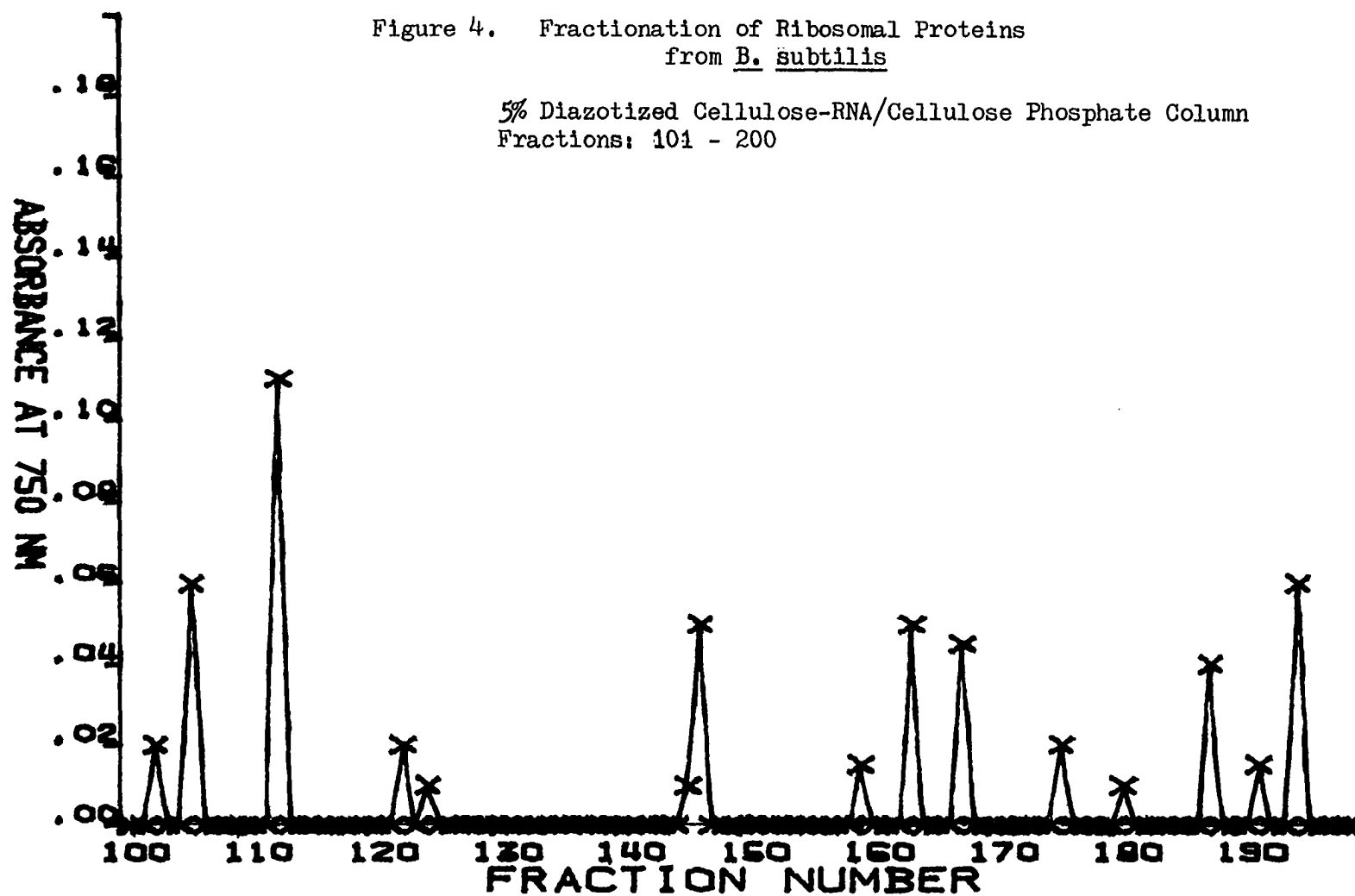
In all of these runs, which take 4 days each, the column was compressed by not more than about 3 cm.

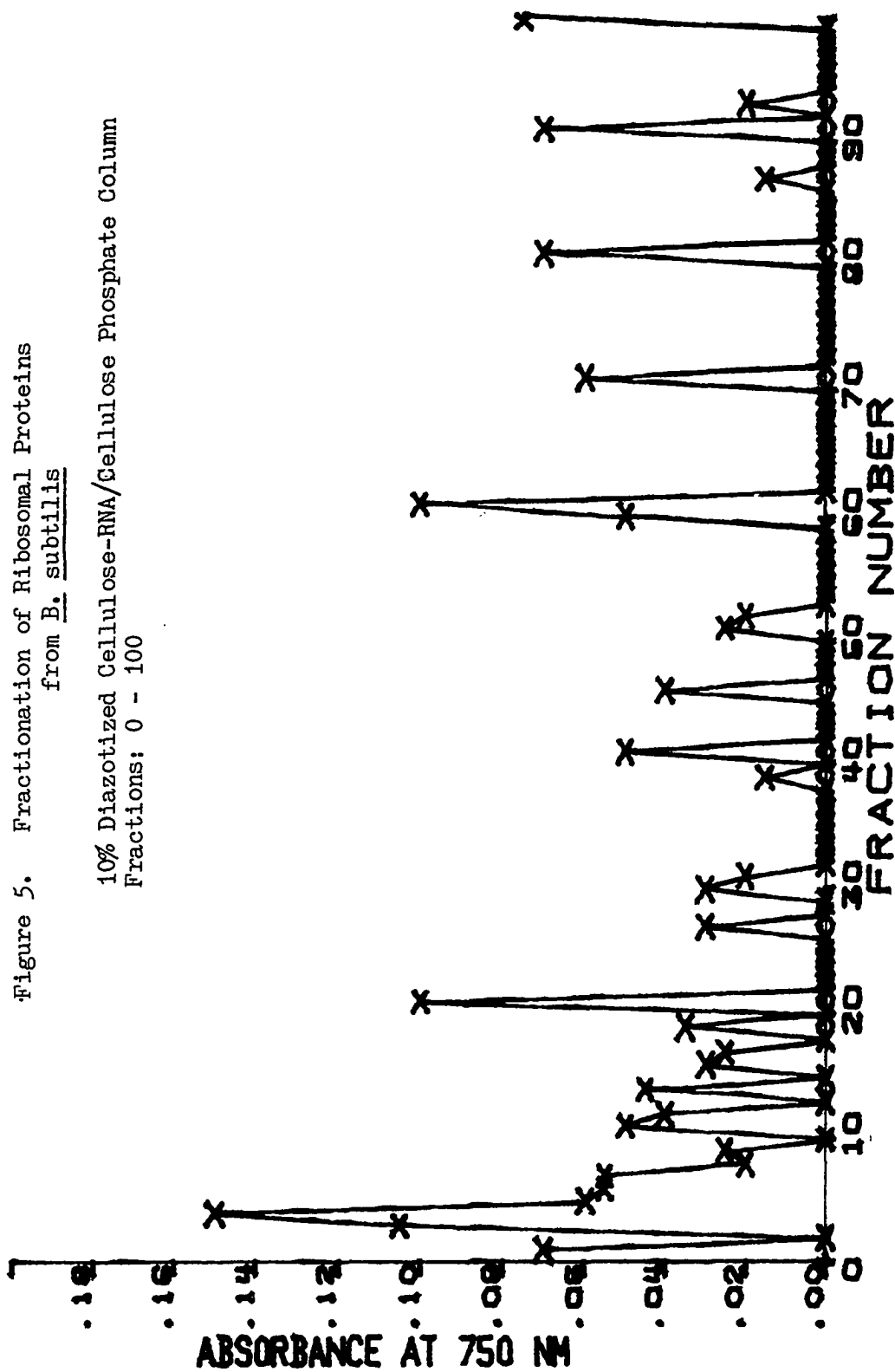
Figure 3 shows a large number of peaks; most of them are not totally resolved (the baseline is above zero absorbance between peaks). Figures 4 and 5 show better resolution and the elution pattern obtained with 10% diazotized cellulose-RNA is similar to that obtained with 5% diazotized cellulose-RNA. Comparing Figures 4 and 5, it appears that there are some differences in both the number of peaks and in their relative amounts. No definite conclusions, however, can be reached regarding these apparent differences because of the following complications. Some protein always precipitated out at the top of column. This prevented a precise analysis of the chromatographic recovery of the applied protein. Moreover, different batches of diazotized cellulose-RNA were used for the two columns. These were prepared using identical conditions but may still differ slightly in their RNA content. In addition, the aliquots of protein applied to the column were weighed out and errors may have been involved at this stage as well (e.g. varying degrees of hydration). Lastly, because of the fact that some protein precipitated out in the column, it was necessary to prepare a fresh column for each run. This was done as reproducibly as possible but falls short of the preferable approach

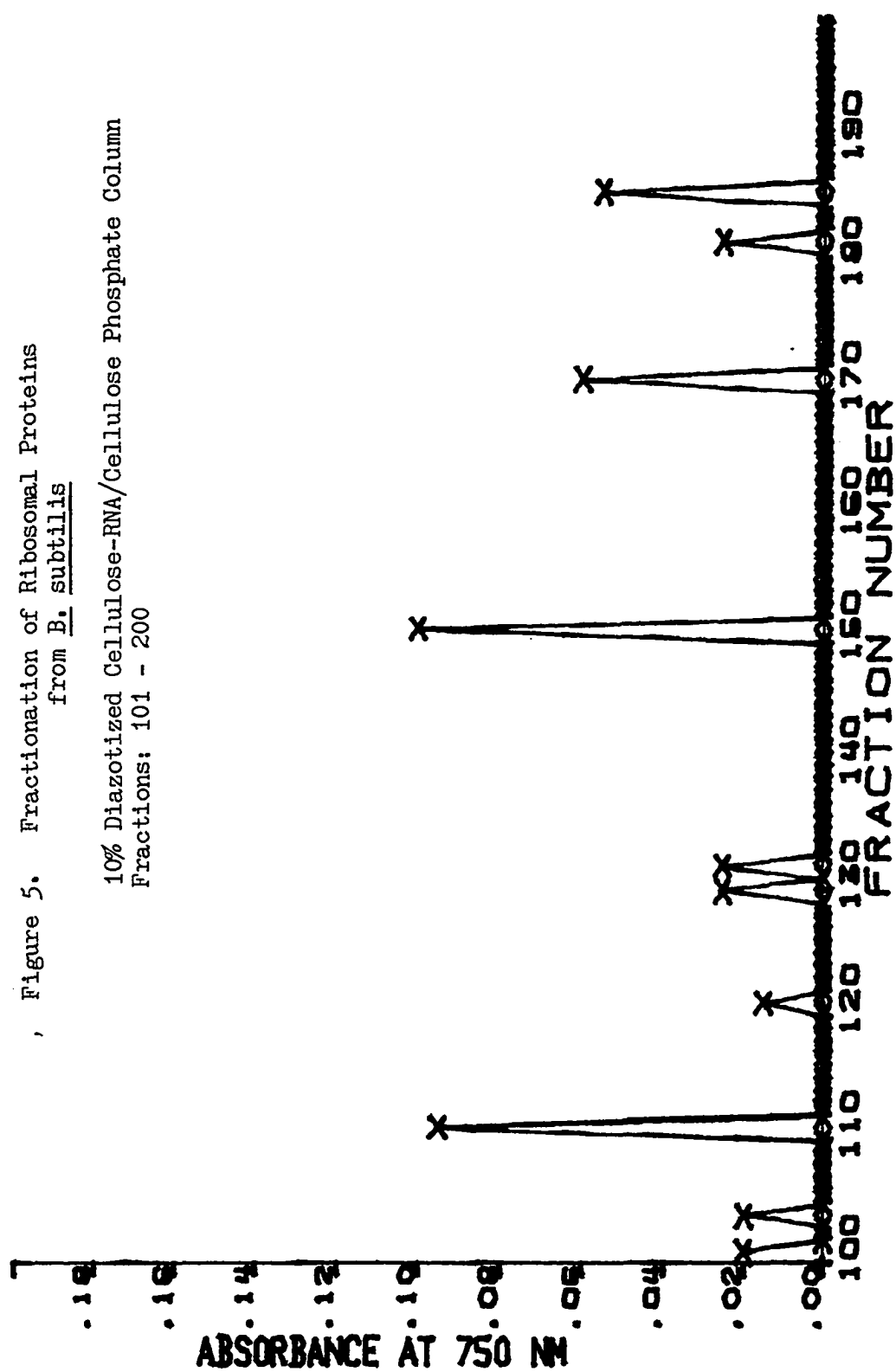












in which one and the same column is used for all of the experiments.

All in all some 30 different fractions were obtained with the 10% diazotized cellulose-RNA out of a theoretical maximum of about 50 (there are some 50 different proteins per ribosome). In the present study, an attempt has been made to develop affinity chromatography as a new method for the fractionation of ribosomal proteins. In order to show that affinity chromatography can be used effectively, it is essential to demonstrate that the elution pattern is both qualitatively and quantitatively representative of the mixture of protein components originally present within the ribosomes. This calls for an identification of the proteins within each fraction; and for a quantitative analysis of the amount of each protein. Such studies as well as further resolution of the first peak (fractions 0 - 10) are recommended for future work. Other concentrations of diazotized cellulose should also be tried. The procedure used here is relatively simple and might prove useful for other studies involving ribosomal proteins. It should also be possible to adapt this method to large scale fractionation of ribosomal proteins.

SUMMARY

Ribosomes were isolated from B. subtilis. Ribonucleic acid was isolated from the ribosomes by phenol treatment. The RNA preparation contained no detectable DNA and protein was absent or, at least present in negligible amount. Ribosomal proteins were isolated by the 2-chloroethanol method with about 0.2% contamination by ribosomal RNA.

Fractionation of ribosomal proteins was studied by affinity chromatography using a cellulose column support to which ribosomal RNA had been covalently attached.

The elution patterns showed a selective and efficient fractionation of the ribosomal proteins. Peaks were well resolved and reproducible patterns were obtained. The fractionation was affected by the concentration of RNA-diazotized cellulose in the column.

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VITA

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