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Fractionation of Ribosomal Proteins from Mesophilic and Thermophilic Bacteria

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FRACTIONATION OF RIBOSOMAL PROTEINS FROM MESOPHILIC AND THERMOPHILIC BACTERIA

by

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INTRODUCTION

Thermophilic organisms live at temperatures (50-80° or higher) which, in vitro, can cause the destruction and denaturation of many macromolecules necessary for life. Because proteins, nucleic acids, and many other biological compounds are denatured at these temperatures, any organism existing at these temperatures must possess unusual mechanisms which permit survival.

Mesophiles live at more moderate temperatures (20-45°) where destruction and denaturation due to temperature are not a problem.

Three theories have been proposed to explain thermophily. The first attributes thermophily to the protective action of lipids at high temperatures (1). The second postulates a special metabolic state characterized by high rates of breakdown and resynthesis (2). The third attributes thermophily to the structure and function of macromolecules and emphasizes physical-chemical differences of macromolecules from mesophiles and thermophiles (3). The third theory has received the most experimental support so far. Previous work from this laboratory in support of this theory has dealt with differences in fatty acids (4), ribosomes (5), ribosomal ribonucleic acids (6), deoxyribonucleic acids (DNA; 7), DNA polymerase (8), cell-free amino acid-incorporating systems from mesophilic and thermophilic strains of the genus Bacillus (9, 10), polysome functions (11), and ribosomal proteins (12).

Studies indicate that cytoplasmic proteins of thermophilic organisms are very similar to their mesophilic counterparts in many physical and chemical properties but that differences associated with metal binding ability and hydrophobic amino acids exist (13).

The heterogeneity of ribosomal proteins has been demonstrated (12, 14, 15, 16, 17, 18, 19). The work on bacterial ribosomal proteins has been limited primarily to Escherichia coli. E. coli ribosomal proteins, although heterogeneous, are similar in many chemical and physical properties, and are relatively insoluble in aqueous buffers (14, 15). Research in this laboratory has shown that ribosomal proteins of the genus Bacillus are likewise heterogeneous (12).

Bacillus ribosomal proteins are very insoluble compared to those of E. coli. Proteins from the thermophile B. stearothermophilus are less soluble than those from the mesophile B. licheniformis; the problems caused by the low solubility in protein analysis by two-dimensional polyacrylamide gel electrophoresis have been described (12).

These studies were undertaken to find suitable solvents for the Bacillus ribosomal proteins and to develop an alternate method to electrophoresis for fractionating ribosomal proteins. Three methods were studied - countercurrent distribution, Sephadex gel filtration, and carboxymethyl cellulose column chromatography. A technique for direct tritium labeling of the ribosomal proteins is also described.

MATERIALS AND METHODS

Preparation of Subcellular Fractions

Cells of B. licheniformis (NRS 243) and B. stearothermophilus 10 were grown, harvested, and stored as described previously (9, 10). The cells were thawed, washed, and broken in a French pressure cell; the ribosomes were isolated from this suspension as described (9, 10), and ribosomal proteins were prepared from the ribosomes according to the procedure of Fogel and Sypherd (14).

Tritium Labeling of Ribosomal Proteins

The reductive methylation (tritium labeling) was patterned after Moore (20).

Ribosomal protein (1.5 mg) was dissolved in 1.5 ml BMK-guanidinium hydrochloride buffer (100 mM sodium borate, 10 mM magnesium chloride, 20 mM potassium chloride, 6 mM β-mercaptoethanol, and 4 M guanidinium hydrochloride, pH 9). Formaldehyde (approximately 37% and containing 10-15% methanol as a preservative; Matheson and Coleman) was refluxed for 1 hr, cooled and diluted to a final concentration of roughly 0.08 M immediately before use. Sodium borohydride reagent was prepared just prior to use by adding 4.4 mg cold sodium borohydride to 100 mC tritiated sodium borohydride (5.9 C/mmole; Amersham) in 0.5 ml distilled water at 4^{o} in a fume hood.

The labeling reaction was carried out in an ice bath in a fume hood; this serves to keep the reaction at 4^{o} and to contain any tritium gas evolved during the reaction (21). A maleic anhydride trap was used

to collect tritium gas not used in the reductive methylation. A 500 ml side arm flask containing 15 gm maleic anhydride, 100 ml absolute ethanol, a small amount of palladium, and a magnetic stirring bar was connected to a water aspirator and placed on a magnetic stirrer. The inlet tube was submerged in the ethanol and connected by latex tubing to a small funnel inverted over the reaction tube. A light vacuum was applied during the reductive methylation reaction. The trap operated at room temperature. Formaldehyde reagent (250 μl) was added to 1.5 ml of protein solution; 30 sec later 100 μl of sodium borohydride reagent were added. After 1 min, the treatment with formaldehyde and sodium borohydride was repeated. The reaction mixture was kept at 4° for 10 min with occasional shaking, and was then dialyzed for 24 hr at 4° against 100 ml of TMA buffer (10 mM trishydroxymethylaminomethane hydrochloride, 10 mM magnesium chloride, 20 mM ammonium chloride and 6 mM β-mercaptoethanol, pH 7.8) with three changes of buffer. The dialyzed reaction mixture was diluted to 3 ml with TMA buffer and stored at -20° in 0.1 ml aliquots.

Countercurrent Distribution

The ability of two or more solutions to form two distinct phases was determined by mixing equal volumes of the solutions in a test tube, allowing them to equilibrate, and observing whether or not two phases were formed. Countercurrent distribution trials were run with the solvent systems using five and ten tube manual transfers in order to test for the partitioning of the proteins and the ability of the phases to remain intact.

Two promising solvent systems were tested using a 30 tube hand operated Craig countercurrent distribution apparatus, commonly referred to as the "Craig apparatus." Operation of the Craig apparatus was described by George Kreick, "Operation of the 200 Tube Craig Countercurrent Distribution Apparatus," (unpublished) and by Craig (22, 23). The two-phase system must meet two requirements: (1) the two phases must form reproducibly upon repeated mixing, and (2) the proteins should be equally soluble or nearly so in both phases (22). Systems tested are listed in the Results section. The contents of the tubes were allowed to settle until two stable and clear phases were formed. This required 10 min initially and lengthened to 25 min during the last 10 stages of the 30 tube transfers.

Sephadex Gel Filtration

Sephadex G-50, G-100, and G-200 medium grain (Pharmacia Fine Chemicals, Uppsala, Sweden) were hydrated in either 6 M urea or 1% sodium dodecyl sulfate (SDS), containing 0.02% sodium azide as a bacterial inhibitor; the "fines" were removed, the solution was deaerated, and a 1.3 x 50 cm column (23 ml void volume) was prepared (24). One ml of a solution of ribosomal protein (1 mg/ml) in either urea or SDS was loaded on the appropriate column and eluted with the solution used to dissolve the protein. The flow rate was 3 ml per hr for the G-50 and G-100 columns and 6.7 ml per hr for the G-200 column. Five ml fractions were collected. The elutions were conducted at 4°.

Carboxymethyl Cellulose Column Chromatography

Sodium acetate elution

Carboxymethyl cellulose (CMC; a cation exchanger) was hydrated overnight at 4° with AcU buffer (0.005 M sodium acetate, 6 M urea, pH 5.6) and treated batchwise with a bovine serum albumin (BSA) solution in water (10 mg/ml) in order to tie up the nonspecific binding sites on the cellulose; the cellulose was then used to prepare a 5 x 60 mm column (5.75 inch pasteur pipette) (19). Silylized glass wool and 3 mm glass beads were used as support. Small bore tygon tubing was used as inlet and outlet tubing. A complete run of eluants was made to clear unbound BSA from the column. The column was then loaded with 100 µl of labeled protein containing approximately 45 µg of protein (2×10^6 dpm) diluted with 10 ml of AcU buffer. A 100 µl aliquot of the diluted protein solution was counted to determine the amount of activity added to the column, and the remaining 10 ml portion was loaded onto the column. The loading was done by pipetting the solution directly onto the column. The pipette was rinsed and the rinse applied to the column as well. The combined volumes were allowed to enter the column and elution was started. The column was eluted with AcU buffer until the dpm in the eluate dropped to those of the background. The elution was continued with 0.006, 0.007, 0.009, 0.0125, 0.018, and 0.030 M sodium acetate solutions in 6 M urea at pH 5.6 (19). Each solution was run until the dpm in the eluate dropped to background level. Four ml fractions were collected. The volume of the column including the inlet and outlet tubing was 4 ml. The acetate

solutions were prepared by dissolving sodium acetate in 6 M urea and adjusting the pH with glacial acetic acid. The elution was carried out at 4°.

Sodium formate-ammonium hydroxide-ethanol elution

CMC was conditioned batchwise successively with 0.1 M NaOH, 1.0 M K_2HPO_4, 1.0 M KH_2PO_4, 50% formic acid, 0.02 M sodium formate (pH 2.7), 0.01 M sodium formate-0.03 M lithium chloride-0.08 M urea (pH 2.7), and BSA solution (10 mg/ml) and then used to prepare 5 x 60 mm columns (25). The labeled protein solution (100 µl containing approximately 45 µg and 2 x 10^6 dpm) was diluted with 10 ml of 0.01 M sodium formate-0.03 M lithium chloride-0.08 M urea (pH 2.7) and the entire volume applied to the column. A 100 µl portion of the diluted solution was counted to determine the amount of activity added to the column and the remaining 10 ml portion was loaded onto the column. The tube was rinsed with sodium formate-lithium chloride-urea solution and the rinse was loaded onto the column. The column was loaded as described previously. The proteins were eluted successively with 0.01 M sodium formate-0.03 M lithium chloride-0.08 M urea (pH 2.7), 0.1 M sodium formate (pH 2.7), 0.02 M sodium formate-0.3 M barium chloride (pH 2.7), formic acid (pH 2), formic acid (pH 1.7), and formic acid (40%). The formic acid solutions were prepared by lowering the pH of distilled water with concentrated formic acid. The pH 2.7 formate solutions were made by lowering the pH of the sodium formate solution of appropriate molarity with concentrated formic acid (25). The column was rinsed with water to remove the 40% formic acid and eluted

stepwise with ammonium hydroxide-ethanol-water solutions of increasing ammonium hydroxide concentration (0.1, 1.0, 10.0, 30.0% v/v) and of increasing ethanol concentrations (20.0, 40.0, 70.0% v/v) for each ammonium hydroxide concentration (Table 1). Twelve ammonium hydroxide-ethanol-water eluant combinations were used. The elutions were run at 4°. Four ml fractions were collected. The column volume including the inlet and outlet tubing was 4 ml.

TABLE 1. Composition of the Formate-Ammonium Hydroxide-Ethanol Eluant Series Used With Carboxymethyl Cellulose Columns.

Eluant Number	Eluant Composition*
1	Sodium formate (0.01 M)-lithium chloride (0.03 M)-urea (0.08 M), pH 2.7
2	Sodium formate (0.1 M), pH 2.7
3	Sodium formate (0.02 M)-barium chloride (0.3 M), pH 2.7
4	Formic acid, pH 2
5	Formic acid, pH 1.7
6	Formic acid (40%)
7	Water - distilled
8	Ammonium hydroxide-ethanol-water (0.1:20:79.9)
9	Ammonium hydroxide-ethanol-water (0.1:40:59.9)
10	Ammonium hydroxide-ethanol-water (0.1:70:29.9)
11	Ammonium hydroxide-ethanol-water (1:20:79)
12	Ammonium hydroxide-ethanol-water (1:40:59)
13	Ammonium hydroxide-ethanol-water (1:70:29)
14	Ammonium hydroxide-ethanol-water (10:20:70)
15	Ammonium hydroxide-ethanol-water (10:40:50)
16	Ammonium hydroxide-ethanol-water (10:70:20)
17	Ammonium hydroxide-ethanol-water (30:20:50)
18	Ammonium hydroxide-ethanol-water (30:40:30)
19	Ammonium hydroxide-ethanol (30:70)
20	Water - distilled

*Composition of the ammonium hydroxide-ethanol-water eluants is v/v/v.

Protein Determination

Protein levels were measured by the Lowry modification of the Folin-Ciocalteau technique (26) or by absorbance at 280 nm when applicable.

Radioactivity Measurement

All the radioactive solutions were counted with a Packard Tri-Carb liquid scintillation counter using a cocktail prepared by mixing 7.75 liters toluene, 757 ml Biosolv (Beckman), and 318 ml Liqui-flor (New England Nuclear; containing 4 gm PPO and 50 mg POPOP per liter). The cocktail will solubilize 0.7 ml of water per 15 ml of cocktail. One half ml of sample was counted in 15 ml of cocktail.

RNA Determination

RNA was determined spectrophotometrically using a modification of Schneider's orcinol method (27). Sample (1.5 ml), 0.15 ml of orcinol reagent (100 mg orcinol/ml in 95% ethanol), and 1.5 ml of $FeCl_3$ solution (1.0 mg $FeCl_3$/ml in concentrated HCl) were mixed and heated in boiling water for 40 min. The solutions were cooled and the absorbance read at 700 nm versus a reagent blank. Ribose (Eastman Kodak) at 100 μg/ml in water was used to prepare a series of standards containing 100, 50, 25, 12.5 and 6.25 μg/ml by twofold serial dilutions in water. The standard solutions were assayed at the same time and in the same manner as the samples.

RESULTS AND DISCUSSION

Protein Isolation

The yield of ribosomal proteins of B. licheniformis and B. stearothermophilus and RNA content as determined by the orcinol technique are listed in Table 2. The proteins contain less than 0.38% (w/w) RNA. 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.

Protein Solubility

Protein solubility studies were undertaken with countercurrent distribution applications in mind. Solvents used in countercurrent distribution must meet two criteria: (1) they must form two phases reproducibly when mixed and allowed to separate, and (2) the proteins should partition more or less equally between the two phases. The minimum amount of protein to be added to the Craig apparatus can be calculated from the formula: $A = 1.25\ CV\ (n)^{\frac{1}{2}}$, where A is the minimum amount of protein to be used in loading the countercurrent distribution train, C is the minimum amount of protein that can be detected by the protein determination used, V is the total volume (both phases) for each transfer tube, and n is the number of transfers (28). As an example, the Folin protein assay can detect as little as 6×10^{-3} mg/ml; if the total volume per tube is 20 ml and 30 transfers are used, then one

TABLE 2. Isolation of Ribosomal Proteins.

Organism	Wet Weight of Cells (gm)	Ribosomal Protein Recovered (mg)	RNA Content (% w/w)
B. licheniformis	100	108	< 0.37
B. stearothermophilus	103	196	< 0.36

component would have to amount to 0.89 mg and 55 components to 48.9 mg. If absorbance at 280 nm was used as the protein assay, then a minimum of 40 mg of BSA/ml could be detected reproducibly. Hence, in counter-current distribution a minimum of 5.8 mg would be needed for one component and 301 mg for 55 components if the protein was evenly distributed throughout both solvent phases.

Solubilities were estimated by placing 8-10 μg of protein in a small test tube, adding 100 μl of solvent, mixing gently, and inspecting the solution visually using transmitted and reflected light. This tests the solubility in the range required by the Folin assay for 55 components. Greater amounts would be required for absorbance measurements. Solvents in which the protein is soluble become clear, those in which the protein is sparingly soluble lead to a fine dispersion of the undissolved protein, and those in which the protein is insoluble cause the protein to clump or form large flakes which do not disperse. The ribosomal proteins were soluble in 2-chloroethanol and sodium hydroxide (2 mM) at concentrations greater than 1 mg/ml, and soluble in urea (6 M) and water saturated phenol up to 1 mg/ml. Other solvents were tested singly, in pairs, and as systems described in the literature. These dissolved only a few micrograms (less than 20-30) per ml. The solvents included ammonium sulfate (40%), chloroform, dichloroacetic acid (0.2%), dextran (10%), ethanol, ethyl acetate, formic acid (pH 1.7 and 2), guanidinium hydrochloride (1 M), isoamyl alcohol, polyethylene glycol, secondary butanol, sodium acetate (0.1 M), sodium chloride (10%), sodium perchlorate (0.6 M), trichloroacetic acid (0.05%), and trishydroxymethylaminomethane (1 M, pH 7.4 and 0.7 M, pH 7.4). The proteins were insoluble in diethyl ether.

Due to the low solubility of the protein, interference of a solvent with the protein assay, or failure of a solvent system to form two stable phases, most of the solvents discussed above were not directly useful for countercurrent distribution of Bacillus ribosomal proteins. The 0.05% trichloroacetic acid in 0.02 M acetic acid (pH 5.75)-2-butanol system (1:1 v/v) suggested by previous workers in this laboratory separated reproducibly into two phases, but protein solubility was not high enough to be of direct use. Solubility in the ammonium sulfate:dichloroacetic acid:n-propanol:ethanol system (29) was not high enough to be useful directly, though the system forms two phases reproducibly; furthermore, ammonium salts precipitate the Folin phenol reagent (26, 28). The 6 M urea-water saturated phenol system (1:1 v/v) will solubilize the proteins at an adequate level, but is not dependable in consistently forming two phases. Phenol interferes with the Folin assay. Sodium dodecyl sulfate (SDS) dissolves adequate amounts of protein but foams when mixed, making its use in multiple transfers difficult. The best solvent, 2-chloroethanol, interferes with the Folin assay at levels higher than 4% (v/v) and no suitable solvent was found that would form two phases with 2-chloroethanol and have equal protein partitioning between the phases. The dextran-polyethylene glycol system (30) performs well as a two-phase system, but only sparingly solubilizes the protein.

Spitnik-Elson (31) reported that the solubility of the ribosomal proteins of E. coli depended on the kind of salt present, the pH, the ionic strength, and the temperature of the solvent.

Trishydroxymethylaminomethane (Tris) buffer (ionic strength of 1, pH 7.4) at room temperature would solubilize up to 17 mg of E. coli ribosomal protein/ml. Sodium chloride (ionic strength of 0.5, pH 7) at room temperature and at 4° would dissolve 1 mg of E. coli ribosomal protein/ml, but the solubility was reduced by 50% at 37°.

The solubility of the ribosomal proteins from Bacillus approaches 1 mg/ml in Tris buffer at room temperature but is much less in sodium chloride. Little of the solubility information obtained for ribosomal proteins of E. coli seems to apply to the solubility of the ribosomal proteins of Bacillus.

An additional problem in the use of the solvents discussed above is the difficulty of determining protein dissolved in these solvents. Attempts to precipitate the proteins out of solution (6 M urea or water saturated phenol) by means of trichloroacetic acid or acetic acid were unsuccessful. Attempts to detect the proteins by thin layer chromatography were partially successful. Thus, when bovine serum albumin (BSA) in sodium hydroxide (600 μg/ml) was spotted on silica gel (Eastman #6060), neutralized in a hydrochloric acid chamber, and sprayed with permanganate-periodate solution (32, 33, 34) it could be detected at a level of 1.2 μg. Protein can be detected in this manner even when it is dissolved in 2-chloroethanol. It cannot, however, be detected in either phase of the equilibrated urea-phenol system due to the color produced by the urea nitrogen, nor in the ammonium sulfate-dichloroacetic acid phase, due to the color produced by the ammonium nitrogen. No interfering color is produced by n-propanol

nor by either phase of the trichloroacetic acid-acetic acid-secondary butanol system. Attempts to detect the BSA (1.2 μg) on the thin layer plates by Ehrlich's reagent, ninhydrin and brom phenol blue (32, 33, 34) were unsuccessful. Because of these difficulties, the method of choice for detecting and determining protein in these solvents was that in which the proteins are tritiated and counted (see next section).

Tritium Labeling of Ribosomal Proteins

Tritiated ribosomal proteins with a specific activity of 8×10^7 dpm/mg were prepared from B. licheniformis and B. stearothermophilus (Table 3). The principal product of the reaction is ϵ-dimethyllysine (20, 21). The degree of labeling is dependent on the paraformaldehyde content of the formaldehyde used, with highest incorporations obtained when the formaldehyde is heated under reflux for one hour just prior to use (20). It is important that all the RNA has been removed prior to the reductive methylation because sodium borohydride reacts nonspecifically with RNA (20). The dialysis fluid was counted periodically and by the third change of buffer the count was at background level, indicating that all of the unreacted or exchangeable tritium had been removed. The dialysis fluid should be monitored each time a reaction is run to be sure that only bound tritium remains in the protein solution.

The reaction can also be carried out with ^{14}C-formaldehyde (20, 21); this would form a product labeled with a stronger beta emitter which would be useful for location of spots on films superimposed on electrophoresis gels (35).

By labeling the proteins with tritium, it became possible to use solvents in which the proteins are only sparingly soluble. Theoretically, 3 μg (2.4×10^5 dpm) of labeled protein could be loaded into the 30 tube Craig apparatus, become evenly distributed throughout both phases, and be detected even with the relatively low efficiency of the scintillation counter for tritium. Likewise, very low concentrations of protein could

TABLE 3. Tritiation of Ribosomal Proteins.

Organism	Ribosomal Protein (mg)	DPM Added	DPM Retained	Protein Specific Activity (dpm/mg)
B. licheniformis	1.5	4.4×10^{10}	1.2×10^{8}	8.0×10^{7}
B. stearothermophilus	1.5	4.4×10^{10}	1.2×10^{8}	8.0×10^{7}

be fractionated by column chromatography and detected either in the eluate or in segments of the extruded column.

The reductive methylation procedure has been shown to produce functional ribosomes of high specific activity (20). Methylation of the proteins does not appear to prevent them from binding with RNA; total reconstitution of methylated 30 S proteins and 16 S RNA to give functional 30 S particles has been demonstrated (20). The proteins could be isolated from separated ribosomal subunits, labeled and studied.

The trap system was not too efficient in collecting evolved tritium; only 0.02% of the unincorporated label was found there. The funnel was used so that too high a vacuum would not be placed on the reaction tube. The system could be improved by replacing the funnel with tubing connected directly to the reaction vessel, thus forming a closed system. The vessel used for the hydration of the sodium borohydride should also be connected to the trap, because hydrogen and tritium are evolved when sodium borohydride reacts with water.

Countercurrent Distribution

Five tube transfers of the tritium labeled protein of B. stearothermophilus using the ammonium sulfate:dichloroacetic acid:n-propanol:ethanol and trichloroacetic acid:acetic acid:2-butanol systems were performed manually. No separation was achieved with the ammonium sulfate system (Figure 1). Essentially all of the protein was found in the upper phase (ammonium sulfate) and none in the lower phase (n-propanol). Separation was achieved with the trichloroacetic acid system (Figure 2). The upper (2-butanol) phase carried 43% of the applied activity in the first tube of the moving front. The first tube of the stationary lower phase (trichloroacetic acid-acetic acid) contained 53% of the applied activity. This system was used in the 30 tube Craig apparatus. The lower phase held 66.7% of the B. licheniformis activity in the first six tubes, and the upper phase carried 33.3% of the activity to tubes 22-28 (Figure 3). Similarly, 65.3% of the B. stearothermophilus activity was found in the first six tubes of the lower phase and 34.7% in tubes 23-29 of the upper phase (Figure 4). This system appeared to have potential; however, by the thirtieth transfer the phases were forming emulsions which were not separating completely even with increased standing time. This causes some phase carryover which results in uneven levels of both phases in all of the tubes. Carryover contamination of the opposite phase is possible, although this was not observed in these experiments.

Figure 1. Five Tube Countercurrent Distribution Profile of B. stearothermophilus Ribosomal Proteins.

Ordinate: DPM x 10^3 per fraction

Abscissa: Fraction number (4 ml per fraction)

(△) Upper phase (ammonium sulfate)

(●) Lower phase (n-propanol)

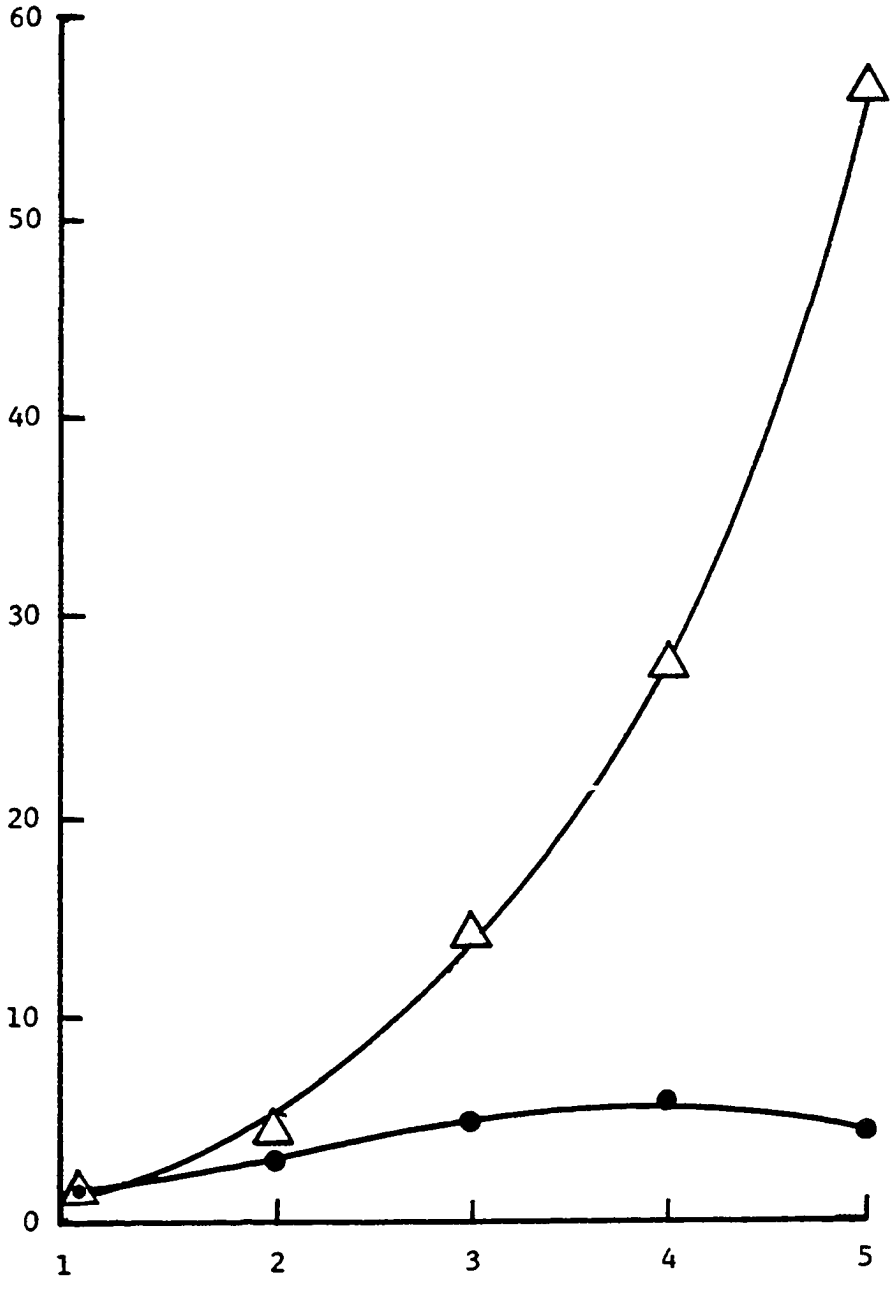


Figure 2. Five Tube Countercurrent Distribution Profile of B. stearothermophilus Ribosomal Proteins.

Ordinate: DPM x 10^3 per fraction

Abscissa: Fraction number (4 ml per fraction)

(△) Upper phase (2-butanol)

(●) Lower phase (trichloroacetic acid-acetic acid)

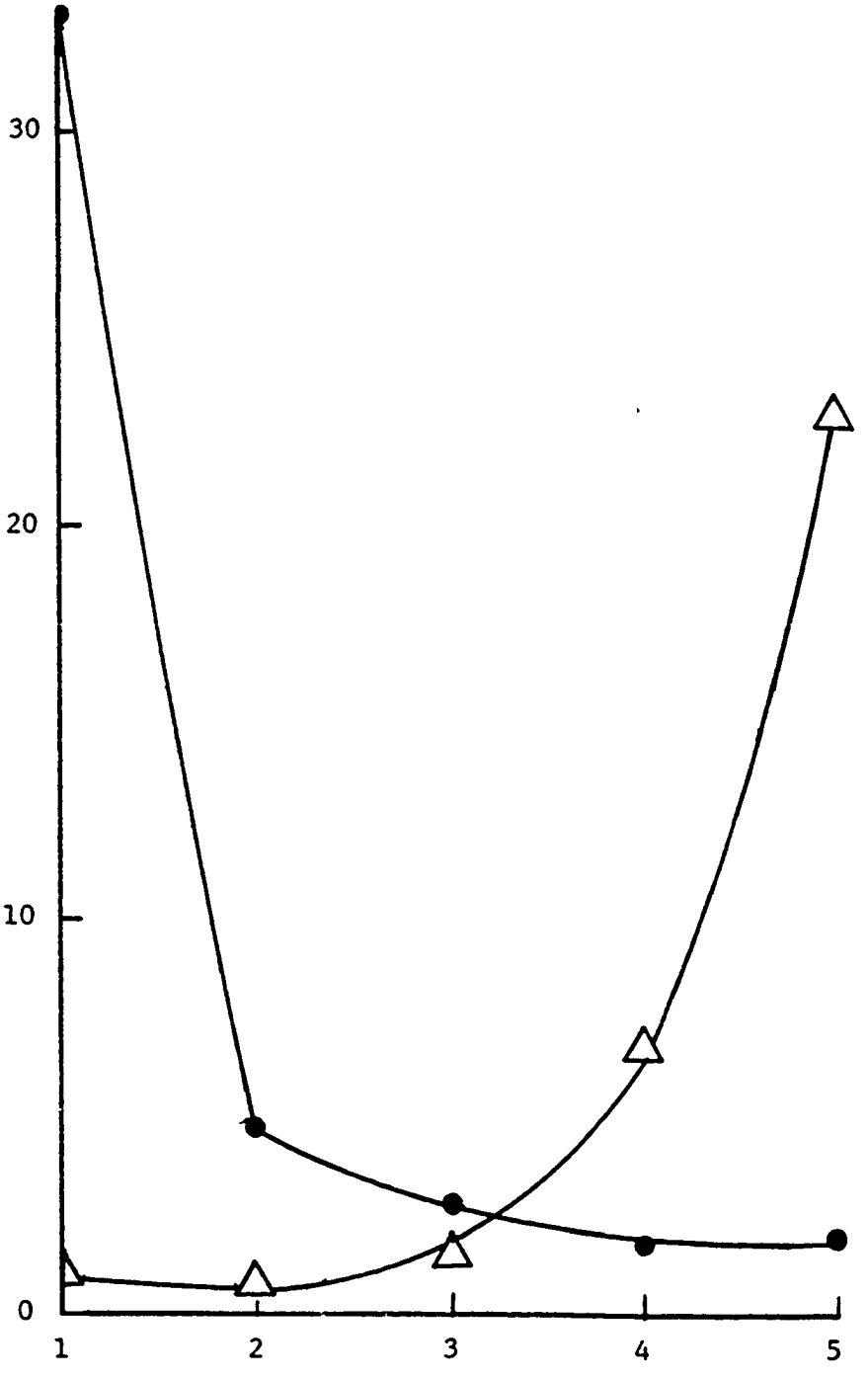


Figure 3. Thirty Tube Countercurrent Distribution Profile of B. licheniformis Ribosomal Proteins.

Ordinate: DPM x 10^4 per fraction

Abscissa: Fraction number (4 ml per fraction)

(△) Upper phase (2-butanol)

(●) Lower Phase (trichloroacetic acid-acetic acid)

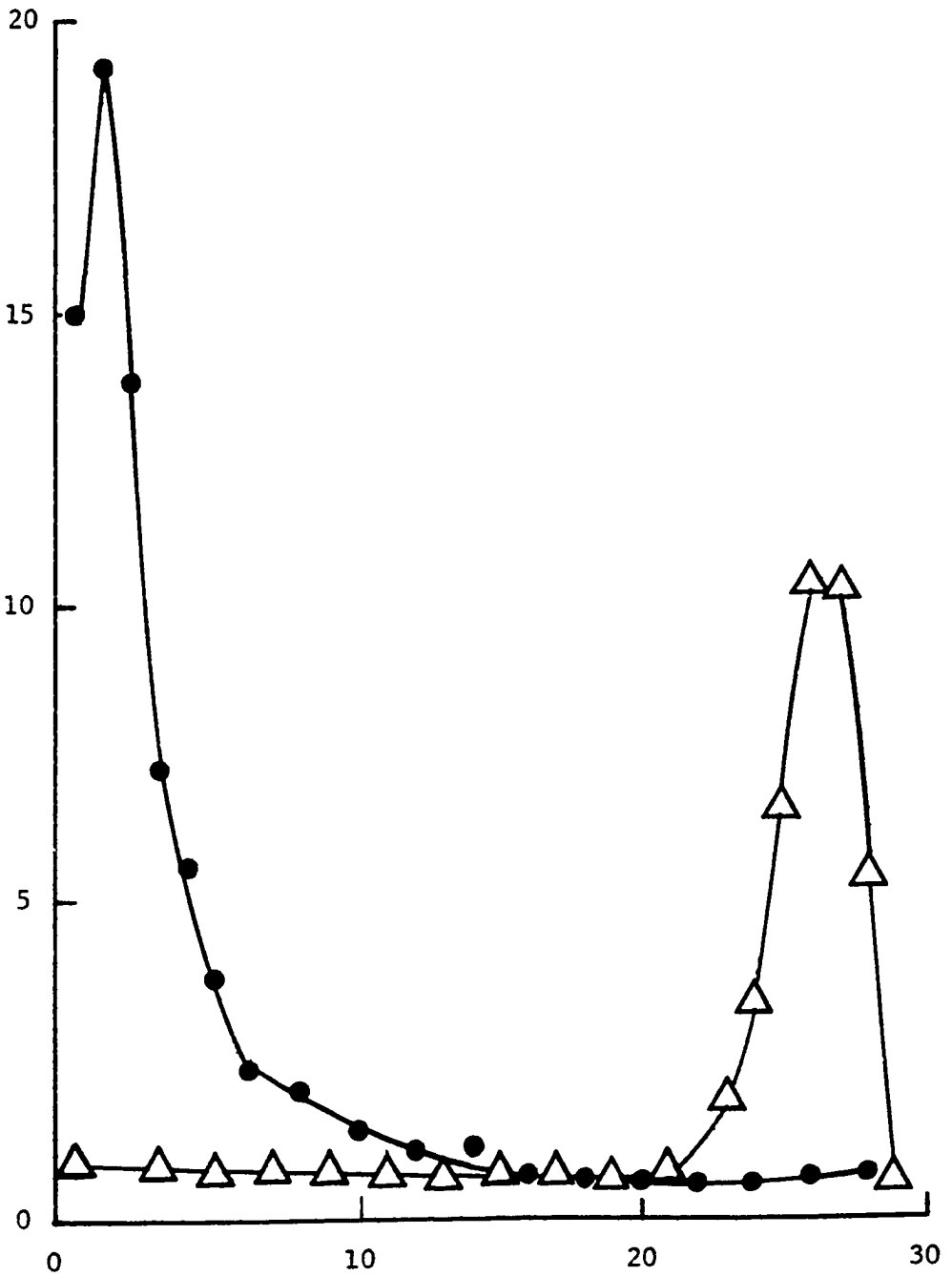


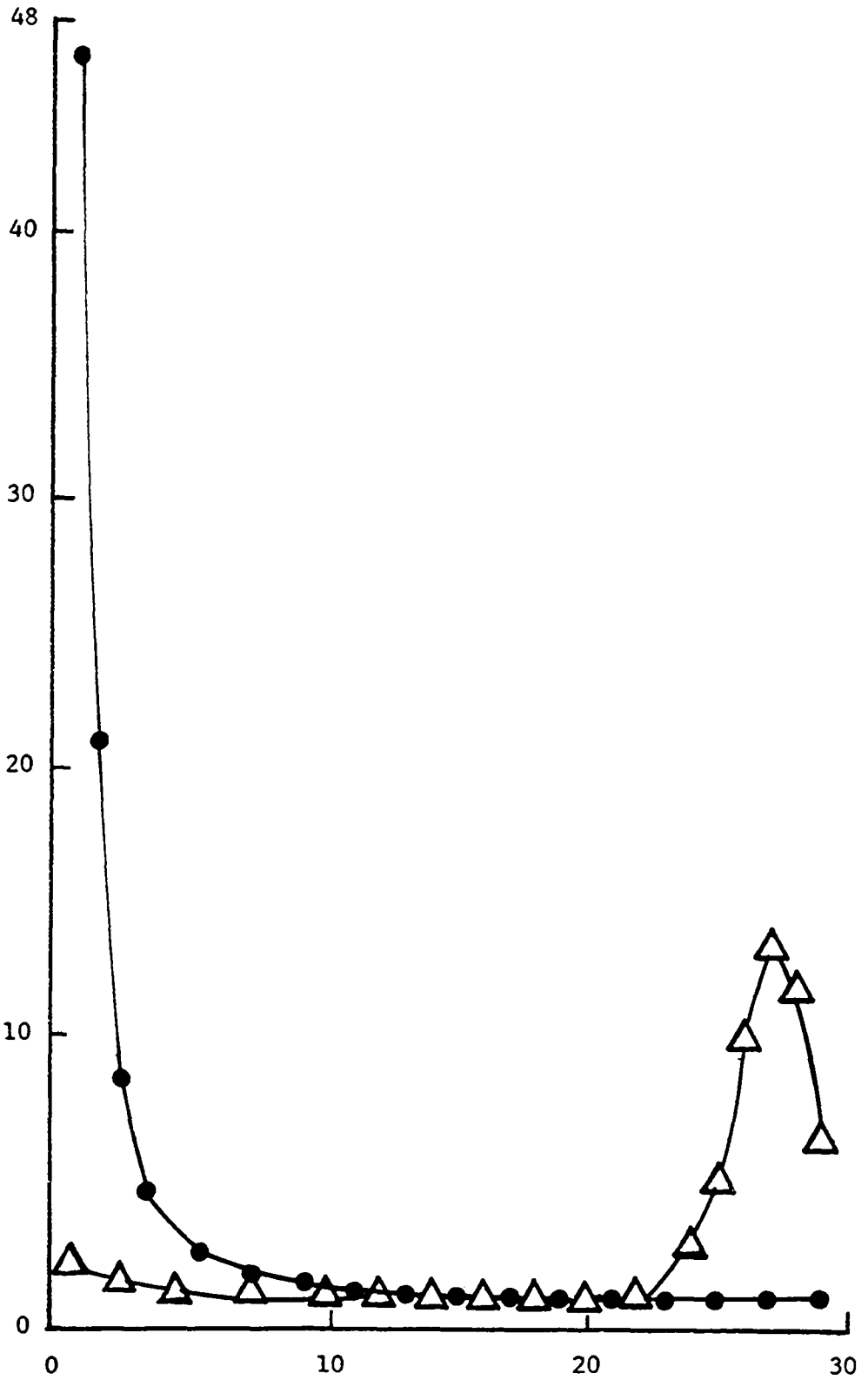
Figure 4. Thirty Tube Countercurrent Distribution Profile of B. stearothermophilus Ribosomal Proteins.

Ordinate: DPM x 10^4 per fraction

Abscissa: Fraction number (4 ml per fraction)

(△) Upper phase (2-butanol)

(●) Lower phase (trichloroacetic acid-acetic acid)



Separation of mixtures of like materials may require only a few transfers if only two components are involved, or several thousand transfers if several components are present (22, 23, 36, 37, 38, 39). If the emulsion problem could be worked out, the n-propanol-ammonium sulfate system could be used in a larger Craig apparatus (200-1000 tube machines) or with high pressure liquid-liquid chromatography.

Due to the difficulties of the countercurrent distribution experiments discussed above, these studies were discontinued in favor of column chromatographic fractionation of the proteins.

Sephadex Gel Filtration

E. coli ribosomal proteins have been separated into molecular weight classes by Sephadex gel filtration using Sephadex G-100 and elution with 6 M urea-acetic acid (40), urea-pyridine-formic acid-mercaptoethanol (17), propionic acid, or propionic acid-urea (15).

In the present study, *Bacillus* ribosomal proteins were found to be insoluble in urea-acetic acid solutions, but to be soluble in urea.

B. licheniformis ribosomal protein was dissolved in 6 M urea and passed through a Sephadex G-100 column hydrated with the urea solution. Over half (56%) of the protein passed through the column in a single peak with the void volume, and little more was removed with continued elution. No protein fractionation was observed. Similar results were obtained with Sephadex G-50 and G-200 eluted with urea, and Sephadex G-50, G-100 and G-200 eluted with 1% SDS. The column eluates were monitored by absorbance (these studies were conducted before the proteins were tritium labeled) and confirmed by the Folin protein assay. These studies were terminated when protein fractionation was obtained with CMC.

The large amount of protein contained in the small loading volume may be too close to the solubility maximum to permit full dissolution and breakup of oligomers or aggregates and may explain the exclusion of the protein from the columns. The columns behaved normally with respect to standardization with BSA and blue dextran (25).

Carboxymethyl Cellulose Column Chromatography

Sodium acetate elution

Waller (19) was able to obtain 93% recovery of *E. coli* ribosomal proteins from CMC columns by stepwise elution with sodium acetate (pH 5.6) from 0.005 to 0.4 M in 6 M urea; he obtained one fraction per step. In the present study, two peaks were obtained when *B. licheniformis* or *B. stearothermophilus* proteins were treated in this manner (seven steps from 0.005 to 0.03 M). The total recoveries were 10% and 14%, respectively, of the activity applied to the column. The results for the *B. stearothermophilus* and *B. licheniformis* proteins are shown in Figure 5. The first peak came through with the loading volume and the second with the 0.007 M sodium acetate eluant. The activity which passed through in the loading volume accounted for 48% of the recovered activity. This technique may have promise if sodium acetate eluants of higher molarity are used; it represents a milder treatment of the proteins compared to that with the formate-ammonium hydroxide-ethanol eluants discussed below.

Sodium formate-ammonium hydroxide-ethanol elution

Carboxymethyl cellulose has been used by itself or preceding or following Sephadex gel filtration to separate and study bacterial ribosomal proteins (16, 17, 19, 25, 40). The solutions from these fractionations were submitted to polyacrylamide gel electrophoresis for further separations and tests of heterogeneity (16, 19, 25, 40, 41).

Figure 5. CMC Column Chromatography of *B. licheniformis* and and *B. stearothermophilus* Ribosomal Proteins - Sodium Acetate Elution.

Ordinate: DPM x 10^4 per fraction

Abscissa: Fraction number (4 ml per fraction)

●——● *B. licheniformis*

——— *B. stearothermophilus*

– – – Denotes eluant change

① Sodium acetate (0.005 M)-6 M urea (pH 5.6)

② Sodium acetate (0.006 M)-6 M urea (pH 5.6)

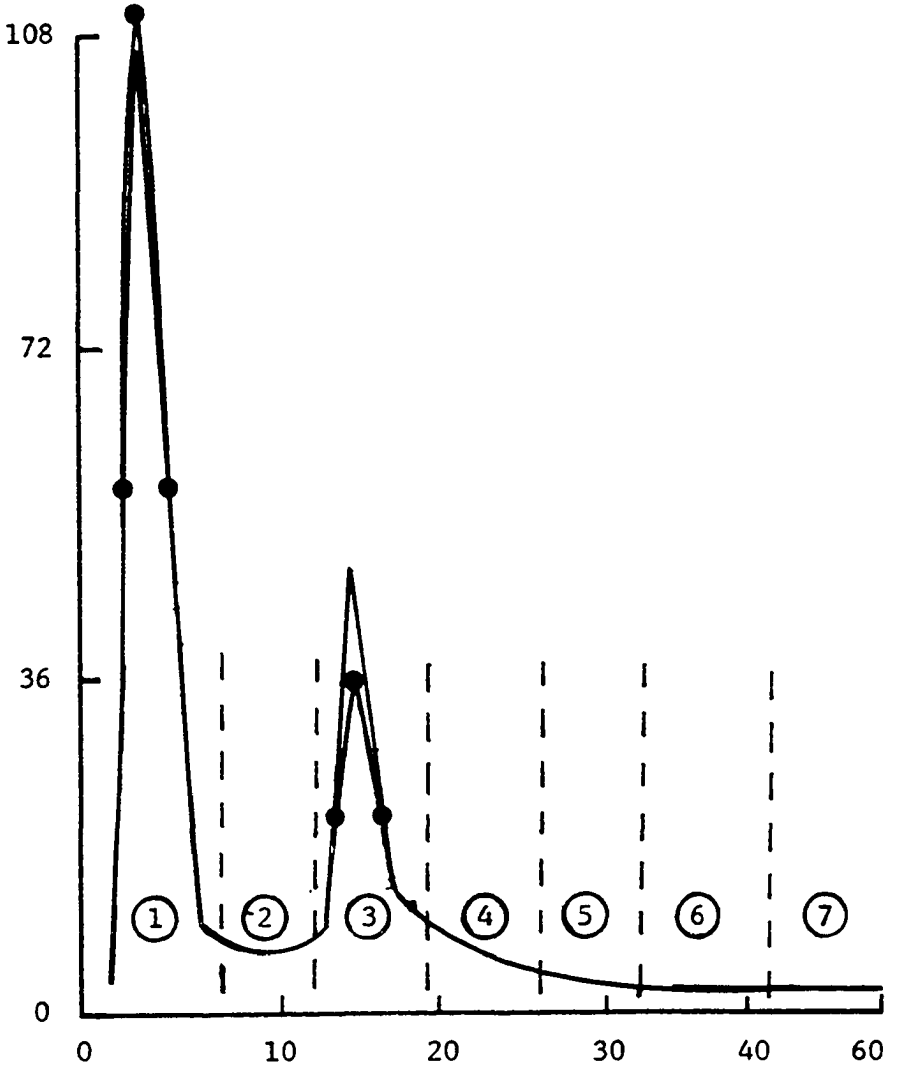
③ Sodium acetate (0.007 M)-6 M urea (pH 5.6)

④ Sodium acetate (0.009 M)-6 M urea (pH 5.6)

⑤ Sodium acetate (0.012 M)-6 M urea (pH 5.6)

⑥ Sodium acetate (0.018 M)-6 M urea (pH 5.6)

⑦ Sodium acetate (0.03 M)-6 M urea (pH 5.6)



Spirin pointed out that fractionation of E. coli ribosomal proteins had been obtained using CMC columns with a variety of eluants (41). Williams had fractionated the ribosomal proteins into six groups recovering 85% of the protein applied to the column (25). Further analysis by gel filtration and polyacrylamide gel electrophoresis demonstrated the heterogeneity of several of these groups.

For the present study, columns were prepared according to Williams (25). B. stearothermophilus protein (1 mg) was dissolved in 1 ml of 0.01 M sodium formate-0.03 M lithium chloride-0.06 M urea (pH 2.7), loaded directly on the column and eluted with the sodium formate and formic acid eluants (see Table 1 in the Materials and Methods section). The eluate was monitored by absorbance at 280 nm. The absorbance measurements were erratic but indicated that some protein separation was achieved. The Folin protein assay was not used because the eluants precipitate the assay reagents or interfere with the color development. Soon after this preliminary work, the proteins were successfully tritium labeled. The labeled B. stearothermophilus protein solution (100 µl containing 44 µg protein and 2×10^6 dpm) was loaded directly onto the column and eluted as described previously. A large amount of activity came through in the void volume indicating that some of the protein was excluded or did not have sufficient time to bind to the column. In an attempt to increase the loading efficiency, 100 µl of labeled B. stearothermophilus protein solution was diluted with 10 ml of the first eluant (sodium formate-lithium chloride-urea) and loaded slowly onto the column. The amount of activity added to the column is given by the equation: $A = C(D-S)/ES$,

where A is the activity (DPM), D is the total volume (ml) of the labeled protein solution plus diluent, S is the volume (ml) of D counted to determine the activity of the loading solution, C is the CPM in S, and E is the counting efficiency. The column was eluted as before. Six peaks, one per eluant, were obtained (Figure 6). The first peak eluted with the latter part of the loading volume; the following peaks were substantially larger than the peaks obtained by loading the undiluted protein solution directly onto the column. The slow loading technique was used for all subsequent experiments. B. licheniformis protein was loaded and chromatographed successfully in the same manner (Figure 6).

It became evident that columns could not be reliably prepared as described by Williams. The 1 M sodium hydroxide conditioning step would often partially or completely digest rather than condition the column. This conditioning step was replaced by conditioning with 0.1 M sodium hydroxide, which proved to be reliable. Protein recovery was roughly the same on columns conditioned with 1 M sodium hydroxide or with 0.1 M sodium hydroxide.

These experiments appeared promising; however, only about 50% of the activity of either protein solution applied to the column had been eluted (Williams claimed a recovery of 85%). A follow-up elution series using the same eluants but without adding more protein to the column produced a similar pattern, but the peaks constituted only 5% of the original peaks and accounted for less than 2% of the remaining protein. This may indicate that a small amount of the types of proteins already eluted remain on the column, but does not account

Figure 6. CMC Column Chromatography of *B. licheniformis* and *B. stearothermophilus* Ribosomal Proteins - Sodium Formate-Formic Acid Elution.

Ordinate: DPM x 10^3 per fraction

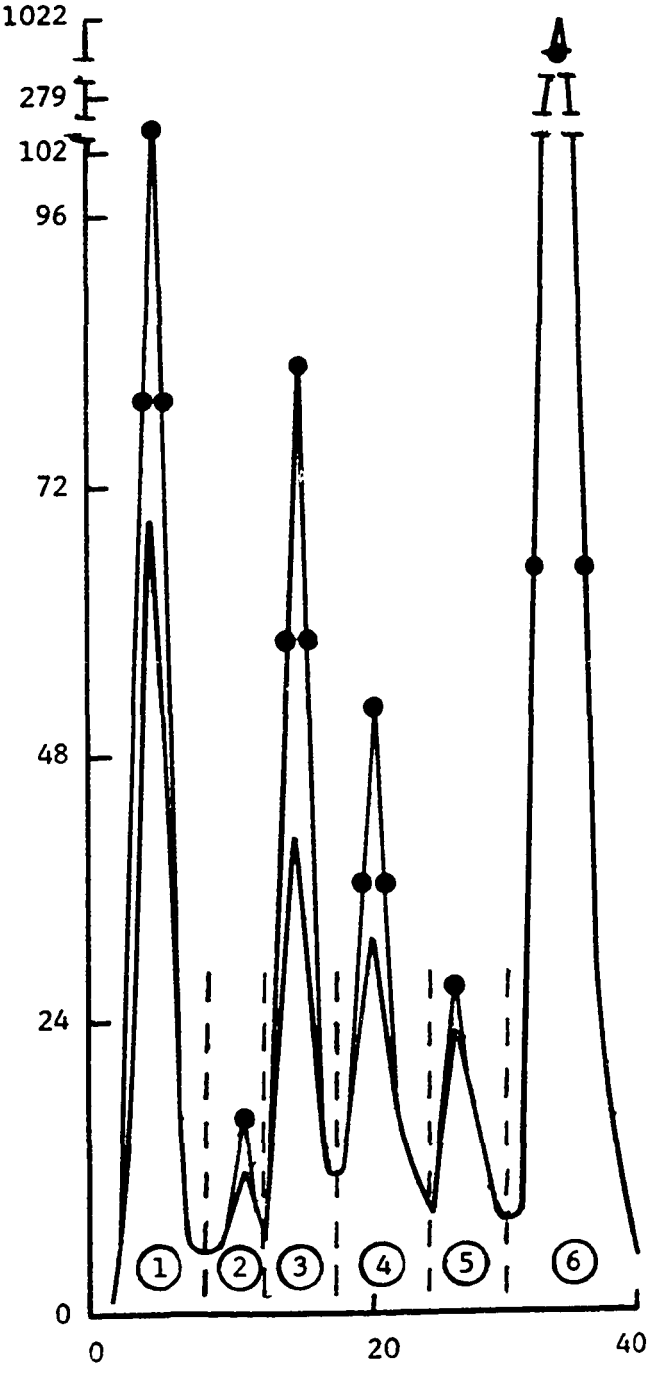
Abscissa: Fraction number (4 ml per fraction)

●——● *B. licheniformis*

——— *B. stearothermophilus*

- - - Denotes eluant change

(1) Eluant number (Table 1)



for the 50% of the protein not eluted. Experiments designed to test additional intermediate eluants were conducted. Fresh columns were prepared and loaded with protein as before. The columns were eluted successively with 0.01 M sodium formate-0.03 M LiCl-0.08 M urea:0.01 M sodium formate (pH 2.7):0.05 M sodium formate (pH 2.7):0.02 M sodium formate-0.3 M $BaCl_2$ (pH 2.7):formic acid (pH 4):formic acid (pH 2): formic acid (pH 1.7):formic acid (20%) and formic acid (40%). The results for the B. licheniformis and B. stearothermophilus proteins are shown in Figure 7. The elution patterns for the two organisms were similar. Instead of obtaining better fractionation than that shown in Figure 6, the result was worse. The recovery was reduced to 30%, and the formic acid peaks (pH 2 and 1.7) were lost.

Other reagents were tested for their ability to release the residual activity bound to the column. Sodium chloride (20 and 30%) released no activity, nor did a water-isopropanol mixture (30:70 v/v). Butanol-glacial acetic acid-water (80:20:20 v/v) also had no effect. Sodium bicarbonate (5%) with a little dry ice added released a large amount of protein, but the carbon dioxide caused gas pockets to form in the column which reduced the flow rate. The urea-pyridine-formic acid-mercaptoethanol mixture (pH 5) used by Hindennach (16) produced no result, but released a small amount of protein when the pH was adjusted to pH 10. Finally, an ethanol-ammonium hydroxide mixture (30:70 v/v) was tried which released a large amount of protein. Similar systems have been used to separate amino acids and their derivatives (32, 33). Following this lead, the ammonium hydroxide-ethanol-water elution scheme (Table 1) was developed and tested.

Figure 7. CMC Column Chromatography of *B. licheniformis* and *B. stearothermophilus* Ribosomal Proteins - Sodium Formate-Formic Acid (Expanded Series) Elution.

Ordinate: DPM x 10^4 per fraction

Abscissa: Fraction number (4 ml per fraction)

●—● *B. licheniformis*

——— *B. stearothermophilus*

– – – Denotes eluant change

① Sodium formate (0.01 M)-0.03 M lithium chloride-0.08 M urea (pH 2.7)

② Sodium formate (0.01 M, pH 2.7)

③ Sodium formate (0.05 M, pH 2.7)

④ Sodium formate (0.02 M)-0.3 M barium chloride (pH 2.7)

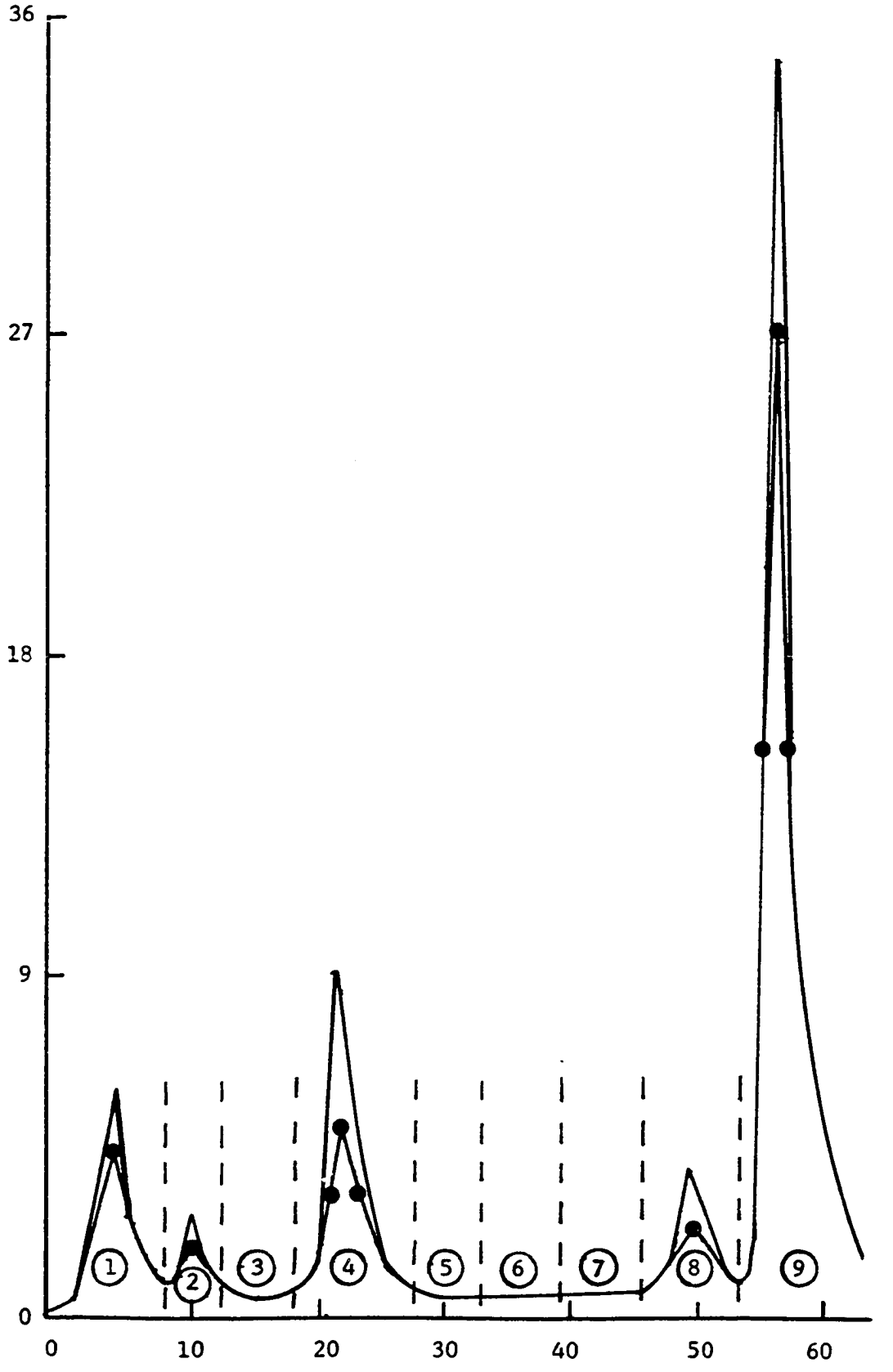
⑤ Formic acid (pH 4)

⑥ Formic acid (pH 2)

⑦ Formic acid (pH 1.7)

⑧ Formic acid (20%)

⑨ Formic acid (40%)



New columns were prepared, loaded, and eluted with the formic acid series followed by a water wash to remove the concentrated acid from the column. The elution was continued with the ammonium hydroxide-ethanol series. The entire run takes two days; the formic acid series requires 4-5 hours, and the remaining time is taken up by the ammonium hydroxide-ethanol series. This is due to the column becoming more and more dehydrated as the concentration of ethanol increases. This reduces the pore openings, which reduces the flow rate. Figures 8 and 9 show the results of the complete elution series (formate-formic acid and ammonium hydroxide-ethanol-water) for B. stearothermophilus and B. licheniformis. Recoveries were 84.2% and 76.1%, respectively. These results were reproducible. The variability between peaks produced by the same eluant in replicate runs was 3-5% with no positive or negative bias. Differences of 5% or less are, therefore, not significant in the comparison of the elution patterns of B. licheniformis and B. stearothermophilus. The amounts in the fractions were expressed as a percent of the total activity recovered from the column (Table 4). The only differences are between fractions 6 and 9. Fraction 6 is 8.7% higher and fraction 9 is 9.7% lower in B. licheniformis than the corresponding fractions of B. stearothermophilus. The first peak may represent protein passing through the column in the 10 ml used to load the column and could be a fraction which does not bind to the CMC or, more likely, protein which did not have time to bind. The peak probably contains some of each of the proteins present in the loading solution.

The columns were taken apart and sectioned into 1 mm sections, each section was dissolved in 15 ml of scintillation cocktail and

Figure 8. CMC Column Chromatography of B. stearothermophilus Ribosomal Proteins - Sodium Formate-Ammonium Hydroxide-Ethanol Elution.

Ordinate: DPM x 10^3 per fraction

Abscissa: Fraction number (4 ml per fraction)

- - - Denotes eluant change

(1) Eluant number (Table 1)

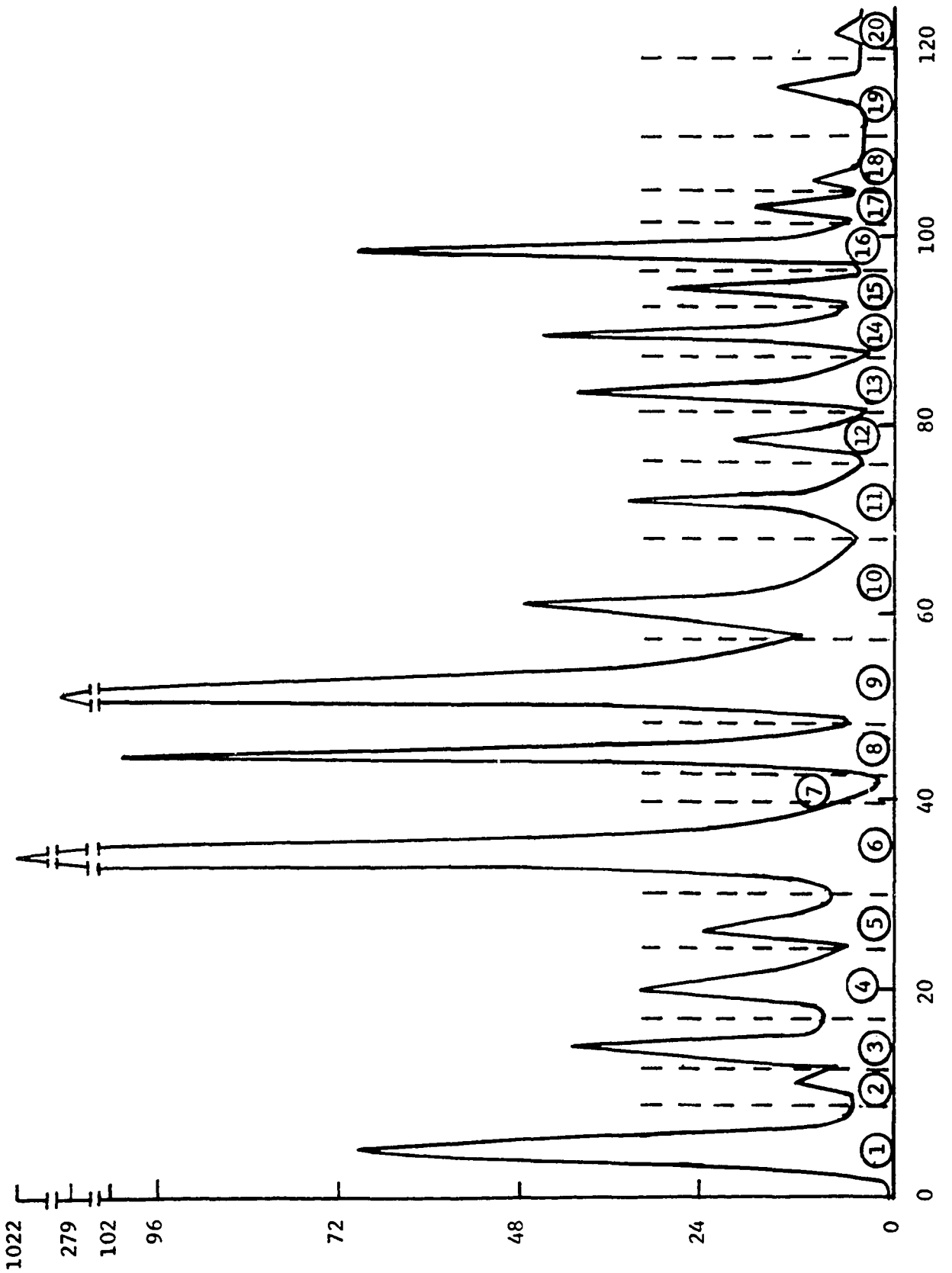


Figure 9. CMC Column Chromatography of B. licheniformis Ribosomal Proteins - Sodium Formate-Ammonium Hydroxide-Ethanol Elution.

Ordinate: DPM x 10^3 per fraction

Abscissa: Fraction number (4 ml per fraction)

— — — Denotes eluant change

① Eluant number (Table 1)

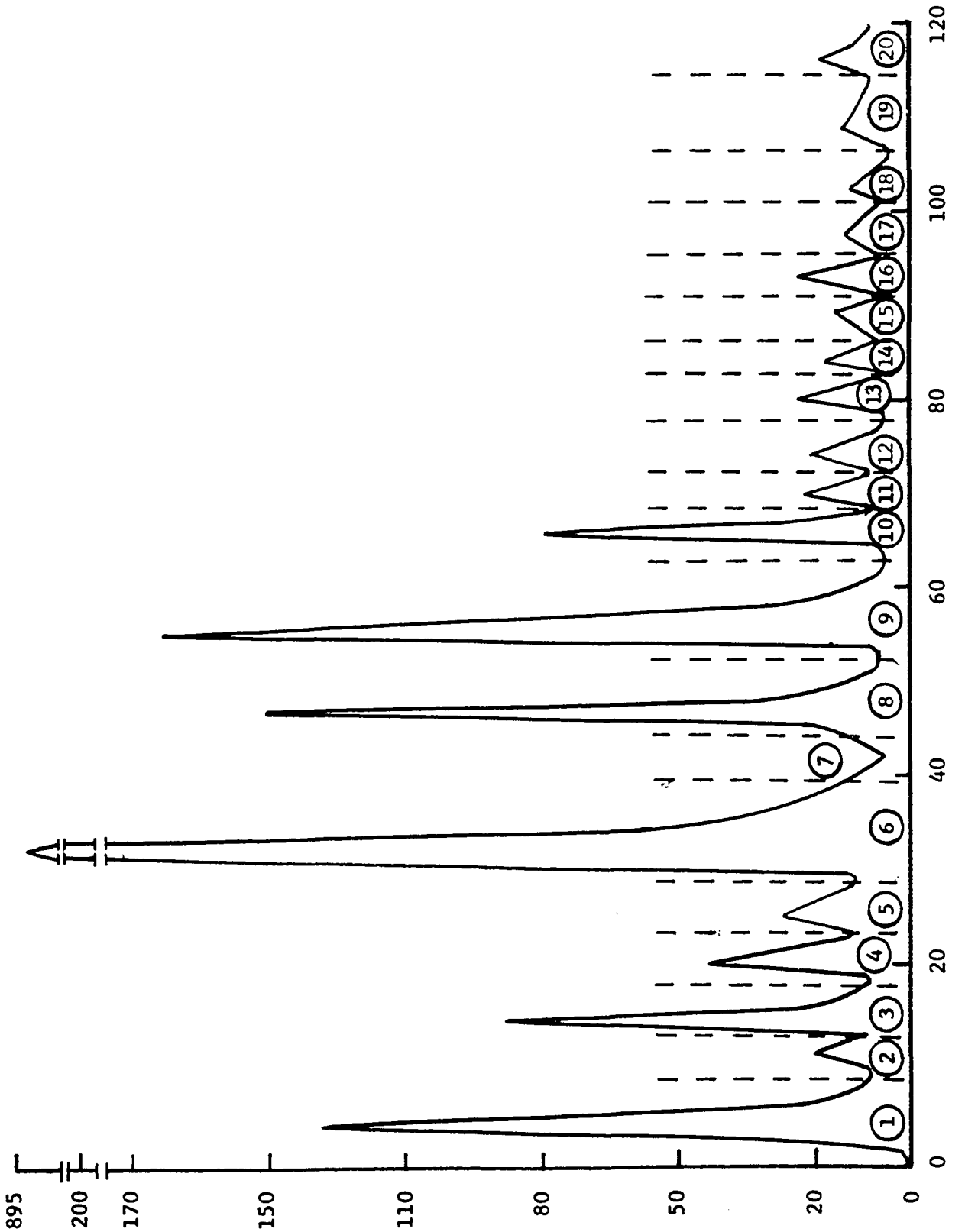


TABLE 4. CMC Column Chromatography of Bacillus Ribosomal Proteins.

	% Recovery*	
Eluant Number**	B. licheniformis	B. stearothermophilus
1	9.1	6.0
2	1.2	0.7
3	3.6	3.5
4	4.0	2.6
5	2.1	2.6
6	50.3	41.6
7	0.1	0.1
8	5.5	4.3
9	10.0	19.7
10	2.8	4.4
11	1.2	1.5
12	1.4	1.1
13	1.1	2.2
14	1.0	2.2
15	1.0	1.1
16	1.2	3.0
17	0.9	1.0
18	0.8	1.1
19	1.7	0.7
20	1.1	0.4

*Expressed as percent of the total activity recovered from the column: B. licheniformis, 4,314,986 DPM and B. stearothermophilus, 3,503,752 DPM.

**Eluant number and composition are listed in Table 1.

counted. The fiberglass wool and glass beads were also counted. The pattern observed was the same for both columns. The top 3 mm of the column contained 70% of the residual activity, and the activity steadily decreased toward the bottom of the column where 1.5% of the residual activity was found in the bottom 3 mm. The lower section of the column contained 19.8% of the residual activity, the beads 0.2%, and the fiberglass wool 10%. The column tube and the outlet tubing were not counted. If they had been, activity would probably have been found there also. In all, 9% of the B. licheniformis and 11% of the B. stearothermophilus activity added to the column was still bound to the column, beads, and fiberglass wool. The activity accounted for was 85% of the activity added to the column for B. licheniformis and 95% for B. stearothermophilus.

Appropriate quench controls were necessary for the sodium formate-ammonium hydroxide-ethanol eluants. Quenching was not marked in any of the eluants; however, the 40% formic acid solution quenched more than the other eluants of the sodium formate-ammonium hydroxide series. The fiberglass wool caused no quenching.

One way to improve the column chromatographic fractionation would be to pretreat the column with the appropriate bacterial protein preparation to tie up all nonspecific binding sites on the column for that particular protein preparation. Several elutions of the same material may be required to fully saturate the nonspecific binding sites. When this condition has been established, a high percent recovery of the material applied is obtained reproducibly. Dr. Satoh (personal communication) has observed this effect with his columns.

This approach is difficult when, as in the present study, only small amounts of the protein preparations are available. The fact that a second elution produces a pattern similar to but 95% lower than that produced by the first elution does not rule out the possibility that proteins entirely different from those eluted may be still attached to the column, but does indicate that proteins like those already eluted are still attached to the column after one elution. It is possible that, with all the nonspecific binding sites tied up, more activity would be obtained with the formic acid elution series and fewer of the ammonium hydroxide eluants would be required. The activity contained in the last peaks of the ammonium hydroxide elution series may represent fragments of protein irreversibly bound by the nonspecific binding sites.

The CMC column technique can be followed by gel filtration and polyacrylamide gel electrophoresis for further separation of the proteins (25).

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