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FRACTIONATION OF RIBOSOMAL PROTEIN FROM *ESCHERICHIA COLI* BY AMMONIUM SULFATE PRECIPITATION*

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SUMMARY

A solution of the total ribosomal protein of *E. coli* has been separated into three fractions by ammonium sulfate precipitation. The solubility and electrophoretic behaviour of the fractions were examined at different pH's at ionic strength 0.02. The fractions differed from each other in both respects. It has been shown that the ribosomal protein contains proteins with widely different isoelectric points, which can interact with one another at low ionic strength to form insoluble complexes.

INTRODUCTION

It has been shown by different investigators[2,3] that the ribosomal protein is not a single protein but a mixture of proteins. In every case in which the ribosomal protein has been subjected to starch-gel electrophoresis, a complex electrophoretic pattern has been obtained; sometimes as many as 20 components were observed[3]. The characterization of the different proteins, however, was hindered because of the rather poor solubility of the ribosomal protein in dilute aqueous salt solutions over a wide range of pH's. We have found that the solubility of the protein increases at higher salt concentrations, and have recently succeeded in solubilizing all of the ribosomal protein of *E. coli* in 1 M Tris buffer[4] (pH 7.4). We proposed that this solubility behaviour might be explained by the presence in the ribosomes of different proteins with isoelectric points on either side of neutrality. At low ionic strength these oppositely charged proteins might interact to form insoluble electrostatic complexes, while high salt concentrations might prevent the formation of such complexes.

Accordingly, we decided to try to separate the ribosomal proteins into a number of fractions—so as to separate some of the acidic and basic proteins and reduce the formation of electrostatic complexes—and to study the solubility and electrophoretic behaviour of each fraction alone. We had, therefore, to start with a solution of the ribosomal proteins in 1 M neutral buffer and to achieve fractionation without lowering the ionic strength. This was accomplished by the step-wise addition of ammonium sulfate to a solution of the entire ribosomal protein of *E. coli* in 1 M Tris buffer (pH 7.4). In this way, we were able to obtain three fractions which showed different solubility and electrophoretic properties.

* A preliminary account of this work has been published[1].

Although the fractions were still heterogeneous, their different properties can explain the solubility and electrophoretic behaviour of the whole ribosomal protein. We were able to show that the ribosomes do contain proteins with isoelectric points several pH units apart, and that at neutrality there are proteins of opposite net charge.

MATERIALS AND METHODS

Preparation of ribosomes

Ribosomes were prepared from exponentially growing cultures of *E. coli* strain W, essentially according to the procedure of TISSIÈRES *et al.*[5], but with the following modifications: DNAase was not added to the crude extract; instead, the extract was diluted to the largest possible volume so as to reduce the viscosity. The medium used throughout the preparation was 0.001 M Tris (pH 7.4)–0.01 M magnesium acetate. The first centrifugation at 78000 × *g* was restricted to 30 min, and the yellow pellet was discarded. After the third and final 2-h sedimentation at 78000 × *g*, the resuspended ribosomes still showed a yellow tinge. Much of this yellow contaminant could be sedimented by a 10-min centrifugation at 12000 × *g*. If not used at once, the ribosomal supernatant was then stored frozen for periods up to a month. Immediately before use, the ribosomes were centrifuged (generally 1–3 times) at room temperature for 10 min at 12000 × *g* until all yellow material sedimented. The yellow material appears to be a non-ribosomal protein contaminant. Such preparations showed an RNA content of 59–61% by weight.

Preparation of ribosomal protein

The ribosome solution was dialyzed at room temperature against a large volume of 1 M Tris buffer (pH 7.4) containing 30 units penicillin per ml solution. Under these conditions the latent ribosomal RNAase became active and digested the RNA of the ribosomes. The products of digestion were dialyzed out and the protein remained in solution. Dialysis was continued at room temperature until no more ultraviolet-absorbing material left the dialysis bag and the amount of the ultraviolet-absorbing material of the dialysates was equal to that of the (fully hydrolyzed) RNA of the ribosomes.

Fractionation of ribosomal proteins

The fractionation with ammonium sulfate was done as summarized in Table I. Each dialysis was carried out with stirring for 24 h at room temperature, with frequent changes of the outside liquid. All the solutions contained 30 units penicillin per ml solution. The dialysis tubing used was Visking seamless cellulose tubing, size 8/32. The centrifugations were done at room temperature in a Servall SS-4 centrifuge at 12000 × *g* for 10 min.

Solubility experiments

The solubility of the different fractions of ribosomal protein was tested in the following manner. The protein solution in 1 M Tris buffer (pH 7.4) was dialyzed at room temperature for 24 h against the solvent to be tested. After 24 h the contents of the bag were quantitatively withdrawn and the bag was washed with the same solvent. The dialysis residue and the wash fluid were combined and centrifuged for

10 min at 12000 × *g* to remove insoluble protein. Soluble protein was determined in a suitably diluted aliquot of the supernatant.

Protein determination

The protein contents were determined by means of the Folin reaction. Usually no Tris buffer was present in the solutions analyzed. However, if Tris buffer was present, its concentration was lower than 0.005 M and the respective blanks contained the same amount of Tris. At higher concentrations Tris interferes with the quantitative determination of the protein.

Electrophoresis experiments

Horizontal starch-gel electrophoresis was performed according to SMITHIES[6]. Only soluble protein was examined. The gels (30 × 2.0 × 0.6 cm) consisted of 15 g of "Starch Hydrolyzed for gel electrophoresis" (Connaught Medical Laboratories, Toronto) in 100 ml of solvent of ionic strength 0.02.

For introduction into gels, 0.5 ml of the sample solution, containing 750 μg protein, was mixed with starch and the thick paste introduced into slots 4 mm wide. The voltage gradient was 6.5 V/cm. Electrophoresis was carried out in a cold room for 24 h.

Staining of the starch gels

After electrophoresis duplicate halves of the gels were stained with either a 0.2% solution of amido black or a 0.2% solution of nigrosin. The solvent for the dyes was methanol – water – glacial acetic acid (50:50:10, v/v). The gels were immersed in the dye solution for 15 min and then washed with the solvent until no more dye left the gel.

RESULTS

Fractionation of ribosomal protein by precipitation with ammonium sulfate

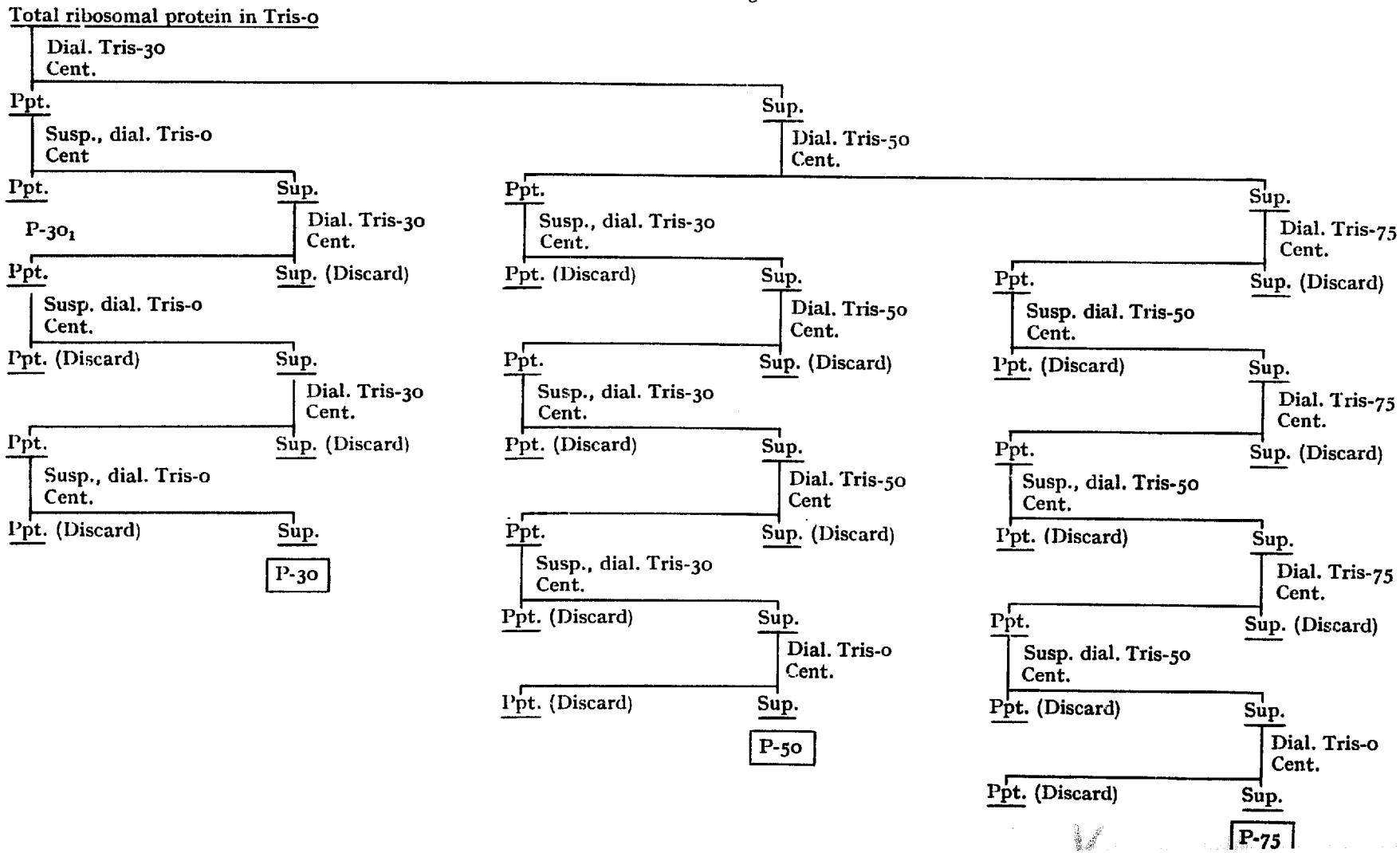
This procedure was chosen in order to achieve a rough fractionation of the ribosomal proteins at high ionic strength, so as to prevent the formation of electrostatic complexes between oppositely charged proteins. The concentrations of ammonium sulfate were chosen arbitrarily to give three sizable protein fractions. The relative sizes of the fractions are indicated by the following experiment. To three separate portions of a solution of ribosomal protein (9.5 mg/ml) in 1 M Tris buffer (pH 7.4) was added solid ammonium sulfate to final saturation levels of 30, 50 and 75%. After 24 h at room temperature, the precipitates were centrifuged down, washed with the appropriate precipitating solvents, dissolved in a solution of NaCl–HCl (pH 2.7, *I*, 0.02), dialyzed against the same solvent to remove ammonium ions, and assayed for protein. The amounts of protein precipitating at 30, 50 and 75% ammonium sulfate saturation, respectively, were 30, 55 and 100% of the total protein. These ammonium sulfate saturation levels were used for the large-scale preparation of the fractions employed in the solubility and electrophoresis experiments; the details are given in Table I.

In the initial stage of the fractionation procedure, the total ribosomal protein, dissolved in 1 M Tris (pH 7.4) is dialyzed against the same buffer 30% saturated

TABLE I

FRACTIONATION OF RIBOSOMAL PROTEINS IN 1 M TRIS BUFFER (pH 7.4) BY PRECIPITATION WITH AMMONIUM SULFATE

The following abbreviations are used: Tris-0, Tris-30, Tris-50, Tris-75: 1 M Tris buffer (pH 7.4) containing ammonium sulfate at saturation levels of 0, 30, 50 and 75%, respectively. Susp: suspended in. Dial.: dialyzed against. Ppt.: precipitate. Sup.: supernatant. Cent.: centrifuged 10 min at 12000 × *g*.



with ammonium sulfate. The precipitate which results does not entirely redissolve in 1 M Tris (pH 7.4) even after 48 h of dialysis against this solvent. The insoluble portion is designated as $P\text{-}30_1$. The soluble portion is called P-30. P-50 is the fraction soluble at 30 % and insoluble at 50 % ammonium sulfate saturation. P-75 is the fraction soluble at 50 % and insoluble at 75 % saturation. All fractions except $P\text{-}30_1$ are of course soluble in 1 M Tris (pH 7.4).

Solubility of the protein fractions as a function of pH

The experiments were performed at room temperature and at a constant ionic strength of 0.02. The pH's tested were 2.7 (HCl–NaCl), 4.2 and 5.8 (acetate buffers), 7.4 (Tris buffer) and 9.4 and 10.6 (carbonate–bicarbonate buffers).

The results (Table II) show that the proteins which precipitate at 30 % ammonium sulfate saturation are highly soluble at the acidic pH's, full precipitation being attained only at about pH 9.5. The fraction P-50 is less basic, with maximal precipitation occurring around neutral pH. The most acidic fraction is P-75, which is least soluble around pH 6. Thus, the fractions differ considerably from one another.

TABLE II

SOLUBILITY OF FOUR FRACTIONS OF RIBOSOMAL PROTEIN AS A FUNCTION OF pH AT I 0.02.

	Protein in solution (mg/ml)				*Per cent soluble*			
Fraction	$P\text{-}30_1$	P-30	P-50	P-75	$P\text{-}30_1$	P-30	P-50	P-75
Starting material	1.50	1.65	1.48	1.50				
pH								
2.7	1.50	1.65	1.48	1.50	100	100	100	100
4.2	1.50	1.50	1.16	1.10	100	91	78	73
5.8	1.25	1.15	0.42	0.11	83	70	28	7
7.4	0.75	0.59	0.14	0.30	50	36	9	20
9.4	0.02	0.18	0.20	0.70	1	11	13	47
10.6	0.08	0.19	0.61	0.92	5	12	41	61

All of the experiments shown in Table II were carried out with solutions containing not more than 2 mg protein/ml, since the solubility seems to be a function of protein concentration. This concentration dependence may well be due to the heterogeneity of the fractions, since each fraction is still a mixture of different proteins. When solubility experiments were carried out at higher protein concentrations, different absolute solubilities were observed. However, the general character of the pH–solubility curves remained and the same differences among the different fractions were observed.

Electrophoretic experiments

Starch-gel electrophoresis was performed as described in the section MATERIALS AND METHODS. The protein solutions in 1 M Tris (pH 7.4) were dialyzed for 24 h against the electrophoresis solvents, which were the same as in the solubility experiments. After 24 h any precipitate found was removed by centrifugation, and the solution was brought to the desired concentration by dilution with the solvent and applied to the starch gel.

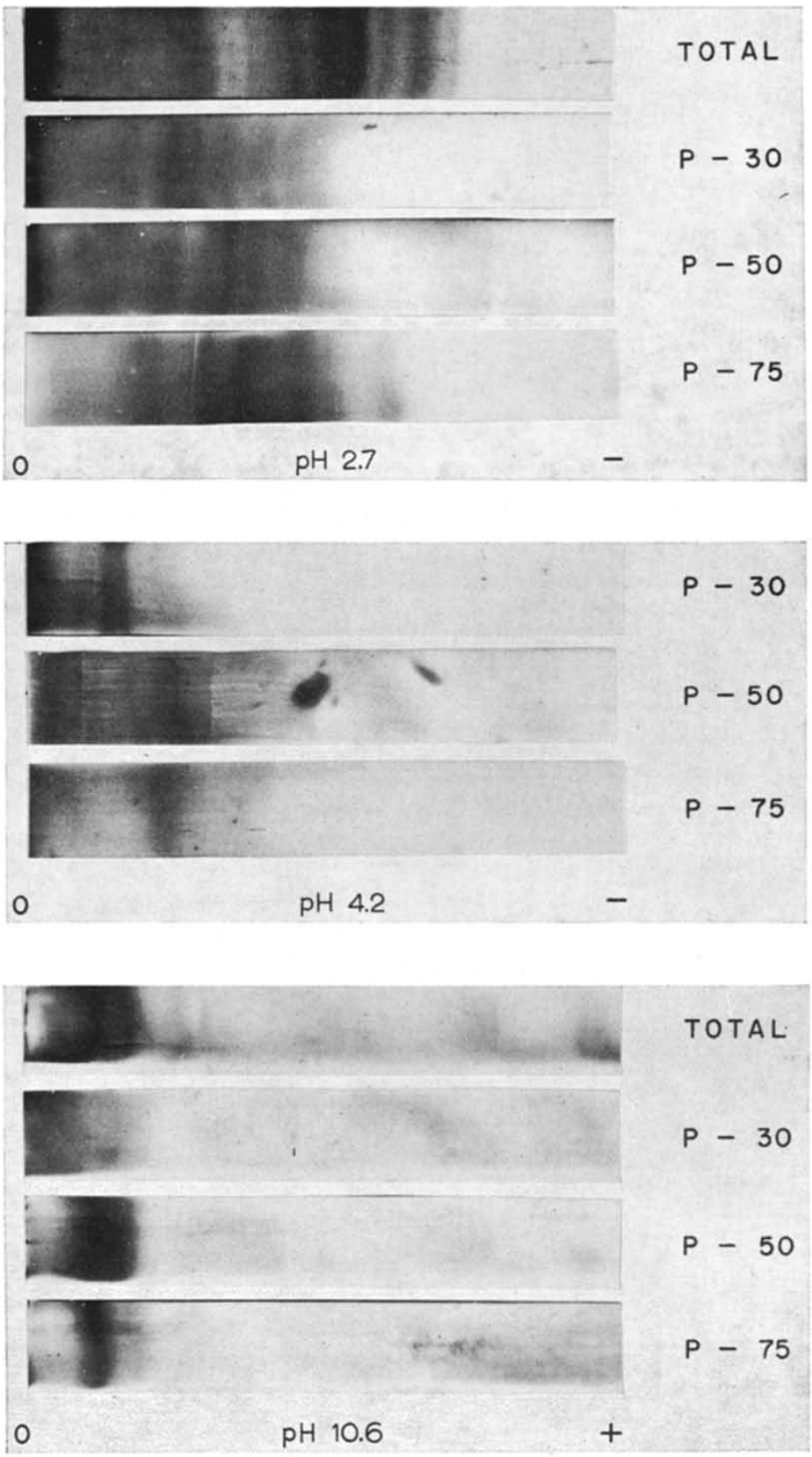


Fig. 1. Electrophoretic patterns of the total ribosomal protein and the protein fractions in starch gel at different pH's. O, origin.

The electrophoretic patterns seen in Fig. 1 are those of the whole ribosomal protein, of P-30_1 and P-30 together, of P-50 and P-75 at I 0.02. At pH 2.7 each pattern contains a large number of bands of different intensities, demonstrating the heterogeneity of each of the fractions. There are bands which are strongly stained and a number of weak bands. The dark bands are different in each fraction, although the concentrations of the protein solutions were the same (1.5 mg/ml). This indicates that fractionation was attained by the ammonium sulfate precipitation; the weak bands which appear in all fractions may, however, indicate some reciprocal contamination.

At pH 4.2 the P-30 fraction shows only two strong bands, while P-50 and P-75 still have a larger number of bands. At both pH's, 2.7 and 4.2, all the protein migrated toward the cathode and none was found to move toward the anode. At these pH's, as well as at all the others which we tried below pH 7, all soluble protein was positively charged and moved toward the cathode. However, at pH 10.6 those portions of P-30, P-50 and P-75 which remained in solution after the removal of the precipitate formed at this pH, moved toward the anode and were, therefore, negatively charged.

Interactions between P-30 and P-75

According to the solubility studies (Table II), the points of minimum solubility of fractions P-30 and P-75 lie on opposite sides of pH 8. That portion of P-30 which is soluble at this pH migrates toward the cathode and is positively charged. On the other hand, the part of P-75 which is soluble at pH 8, is negatively charged and migrates toward the anode.

When these two clear solutions, each at pH 8 and I 0.02, were mixed, a precipitate was formed, an interact between these proteins of opposite net charge.

DISCUSSION

The fractionation reported in this paper indicates strongly that the heterogeneity of ribosomal protein which has been described in the literature is not an artifact resulting from the extraction of the protein with acid[2,3] or detergent[7]. In the present case, the ribosomal protein was brought into solution through the action of the ribosomal RNAase on the ribosomal RNA at neutral pH, and again was found to be unmistakably heterogeneous.

The fractions obtained through ammonium sulfate precipitation have been shown to be different from each other by the two criteria of solubility and electrophoretic behaviour. The fractions are very likely impure, since two reprecipitations are probably not enough to eliminate all cross contamination. Nevertheless, the fractionation was extensive enough to allow a clear demonstration that the ribosomal protein is a mixture of proteins with isoelectric points quite far apart. The isoelectric point of the fraction which precipitates at 30 % ammonium sulfate saturation is above pH 9, while that of the fraction precipitating at 75 % saturation is around pH 6.

Considering that the basic fraction P-30 constitutes only about 30 % of the total protein, one cannot say that the ribosomal proteins are basic. Indeed, SPAHR's[8] amino acid analyses of the ribosomal protein of *E. coli* show the quantity of basic amino acids to exceed the amount of acidic amino acids by only a very little. Since the ribosomal protein is not a single protein, it may contain acidic proteins as well

as basic ones. As we have pointed out before, this would be in keeping with our results and with what is known in general of the behaviour of the ribosomal proteins. It would be interesting to know the relative amounts of acidic and basic amino acids in the different fractions. However, such information will be much more meaningful when obtained on pure fractions isolated from the ribosomes. The method described in this paper is crude and is only the beginning of a study of the fractionation of the ribosomal proteins. A further fractionation of the ammonium sulfate fractions by chromatographic techniques might give pure fractions which could be fully characterized.

We have learned, however, that the ribosomal proteins interact with each other, that they have widely separated isoelectric points, and that they therefore precipitate partially over a wide pH range at low ionic strength. This would appear to explain to a large degree the well-known lack of solubility and the difficulty in the isolation of the ribosomal protein.

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