

SOME PROPERTIES AND FUNCTION OF RIBOSOMAL PROTEINS FROM  
THE SEEDS OF PINUS LAMBERTIANA

by

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## I. INTRODUCTION

Although there has been rapid progress in the delineation of the step by step process of protein synthesis, the ribosomal molecular mechanism on which these processes are dependent is poorly understood. Such poor understanding might be partly due to our lack of understanding of the molecular architecture of the ribosome and partly due to our ignorance about the physical factors involved in the molecular mechanism of the ribosome. Any further sound progress on the understanding of the mechanism of protein biosynthesis on ribosomes will be hampered without having well-defined knowledge on the molecular architecture and physical factors involved in the molecular mechanism of ribosome. As the most characteristic feature of the relation, structure-function, of the ribosome might be cited (1) quantal structure--quantal biosynthetic process of proteins and (2) unsymmetrical structure--unidirectional protein biosynthesis process. Structural studies required for such understanding may rely on the investigation of the nature and organization of the two main constituents, ribosomal proteins (r-proteins) and ribosomal ribonucleic acids (r-RNA). RNA has been known to exist in a few distinct molecular structural transition forms and it has been speculated that step by step protein biosynthesis might be associated with the molecular structural transition of these distinct forms through stabilization interactions with the structural r-proteins. More likely r-protein groups might exist in distinct molecular transition forms and function as direct binding sites or function as an indirect active sites, such as allosteric sites, which could drive protein synthetic

processes in the correct direction. In this sense, we can assume r-protein may be serving as both an isotropic and an anisotropic active transport mechanism in the molecular mechanism of protein biosynthesis. Thus, r-protein may serve not only in the maintenance of RNA conformation but also in the induction of RNA conformational transitions in the protein biosynthesis processes which occur on the ribosome. Some structural ribosomal proteins can be easily dissociated from the ribosome without affecting the integrity of the ribosomal structure and function. Therefore, we can speculate that these dispensable r-proteins may be associated with or dissociated from the ribosome at discrete steps of protein biosynthesis. This would indicate that there may be distinct ribosomal transition forms, formed by the absence or presence of some detachable r-proteins, which determine the functional states of the ribosome in the protein biosynthetic process.

The functional heterogeneity of r-proteins was demonstrated by several research groups, which have shown that specific fractions of the r-proteins are essential to in vitro protein synthesis (Hosokawa et al., 1966; Traub et al., 1966; Staehlin and Meselson, 1966; Fujimura and Nomura, 1966; Raskas and Staehlin, 1967; Traub et al., 1968; Traub and Nomura, 1968). It was shown that peptidyl-transferase is an integral part of the 50S subunit (Traut and Monroe, 1964; Zamir et al., 1966; Maden et al., 1968; Monroe et al., 1968). As some other demonstrations of functional heterogeneity of r-protein, certain resistance of bacteria to antibiotics is associated with altered r-protein components (Leboy et al., 1964; Krembel and Apirion, 1968; Tanaka et al., 1968). Tanaka and Kaji (1968) again found that protein from a 23S core particle,

derived from 30S subunits of dihydrostreptomycin sensitive E. coli strain was responsible for the sensitivity of ribosomes to dihydrostreptomycin. Functions of r-proteins as certain specific enzymes are very obscure. Hardy and Kurland (1966) denied the possibility of putative ribosome enzymes such as polyriboadenylic acid (poly A) synthetase to be structural elements.

Techniques used for the investigation of the function of structural r-proteins are: (1) to remove a fraction of the protein from the ribosomes, leaving inactive ribonucleo-protein cores, and subsequently to reconstitute the functionally active ribosomes from these inactive components--analysis of defective artificial components would give clues as to the function of those missing proteins; (2) to utilize protein derivatizing reagents, which will interact selectively with specific group of r-proteins, and investigate any altered ribosomal function due to the derivatization of the r-protein; (3) to prove the site of action of specific antibiotics. When the site of action of the antibiotics is known, then the identification of the r-protein(s) involved in that site of function in mutant strains resistant to the action of the antibiotics is accomplished by identifying the protein altered as a result of mutation. In the present work, dansyl chloride (1-dimethyl-aminonaphthalene-5-sulfonyl chloride) dispersed on celite was used as a protein reagent. Reasons for choosing this reagent are outlined below: (1) RNA is not dansylated and the protein is selectively derivatized. (2) Dansyl chloride-celite reagent does not include any solvent which might affect the ribosome. (3) The buffer system suitable for the maintenance of

ribosome integrity can be used as the reaction medium. (4) The reaction can be completed in very short period, thus excluding the probability of denaturation of the ribosome by prolonged reaction period. (5) With proper pH of the medium, specific proteins can be dansylated selectively according to their ionization constants. (6) The dansylation reaction can be carried out in the cold, preventing ribosome denaturation. (7) Dansylated ribosomes have strong fluorescence, which can be used as a very sensitive marker. (8) Physicochemical investigation of the fluorescence, for example, quenching of fluorescence, energy transfer, polarization of fluorescence, relaxation time technique, etc., can be performed for further study of the ribosomal molecular mechanism. No ideal reagents and techniques for studies of r-protein function have been reported.

Ribosomal proteins from all sources studied have been found to be very heterogeneous--16-54 different components for 70S and 80S ribosomes and 12-22 components for the 30S subunits. In contrast, small viruses have symmetrical structure and homogeneous coat proteins. The most frequently used method to test the heterogeneity of r-proteins is electrophoresis at acid pH in urea, using starch or polyacrylamide gels. Under these conditions r-proteins are basic and migrate toward the cathode. However, in such electrophoretic techniques, the migration of protein bands is affected by the molecular size, shape and charge. Techniques used for each of these factors of heterogeneity are as follows:

- 1) For the evidence of size heterogeneity
  - a) Lack of equivalence between the number average molecular weight ( $\bar{M}_n$ ), weight average molecular weight ( $\bar{M}_w$ ), and z-average

molecular weight ( $\bar{M}_z$ ) (Möller and Chrambach, 1967; Hamilton and Ruth, 1967; Setterfield et al., 1960; Wolfe and Kay, 1966)

b) Polydispersity upon sedimentation analysis (Setterfield et al., 1960)

c) Different degree of band spreading depending on the pore size of polyacrylamide gel in the disc gel electrophoresis (Möller and Chrambach, 1967). This also can be used for the evidence of conformational heterogeneity

2) For the evidence of charge heterogeneity

a) Isoelectric focusing electrophoresis (present work)

3) For the evidence for chemically distinct entities for the separated r-proteins. The heterogeneity of r-proteins can be verified definitely through this method and several laboratories have started on this work (Kaltschmidt et al., 1967; Fogel and Sypherd, 1968; Moore et al., 1968; Hardy et al., 1968; Craven et al., 1969).

a) Amino acid compositions are distinctive

b) Peptide mappings are distinctive

c) Chemical (from amino acid composition) and physical (from sedimentation equilibrium analysis or SDS gel analysis) molecular weight are in agreement and distinctive.

Besides polyacrylamide or starch gel electrophoresis, other techniques were used to show physical heterogeneity, e.g., immunological method (Fogel and Sypherd, 1968; Traut et al., 1970),  $^{35}\text{S}$ -finger printing (Traut, 1966), chromatographic techniques (Waller, 1964; Spitnik-Elson, 1963), ion exchange techniques (Waller and Harris, 1961; Waller, 1964; Leboy et al., 1964), detachment of r-proteins by salt (Ito and Osawa, 1968;

Spitnik-Elson and Atsmon, 1969; Spitnik-Elson, 1963), column chromatography on carboxymethyl cellulose, and acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Traut, 1966; Moore et al., 1968; Delius and Traut, 1969). The number of r-proteins in ribosomes has never been reported without being accompanied with "at least", "approximately" etc. This fact may well reflect difficulties in distinguishing between extraneously absorbed proteins and structural r-proteins, as well as the possibility of the removal of proteins during purification of the particles. This situation may be further complicated by the presence of dispensable (non-essential) and indispensable (essential) r-proteins. One may pose the question: How are RNA and protein organized in the native ribosome, and how are they distributed between the surface and interior? There is general agreement that the proteins are associated with the nonhelical, rather than helical segments of the RNA, while there is some controversy about the distribution of protein and RNA between the surface and interior. Experimental observations, on which the views about the protein-RNA associations are based, may be summarized as follows: (1) Temperature-absorbance profiles of the ribosome do not differ appreciably from those of the free RNA (Cotter et al., 1967; Schlessinger, 1960). (2) The close similarity of the optical rotatory dispersion curves, ultraviolet-absorption spectra, and/or ultraviolet circular dichroism of RNA in the free state and in the ribosome (McPhie and Gratzer, 1966; Blake and Peacock, 1965; Sarker et al., 1967; Cox, 1969; Wolfe and Kay, 1969). (3) With the addition of extraneous basic protein to the ribosome, a stabilization of RNA is observed, based on the displacement of

the melting curve towards higher temperatures. This is interpreted as binding of the added protein to the double helical regions; suggesting that these double helices are not associated with the ribosomal protein (Daya and Gratzner, 1969). (4) Conformational changes by the treatment with formaldehyde, or by ribonuclease digestion of the RNA moiety in the ribosome are accompanied without alteration of either the sedimentation coefficient or the appearance of the ribosome in the electron microscope. These results also provide further evidence that the spectrum of the RNA moiety does not alter significantly on dissociation from r-protein.

Three general methods of extracting the r-protein from the ribosome are: (1) Self-digestion or RNase (ribonuclease) digestion: ribosomes are dialyzed against an appropriate buffer. RNA is degraded and the degraded products pass through the membrane, leaving the proteins in the dialysis bag. (2) Use of LiCl-urea. (3) Use of acetic acid. In the last two cases, after certain period of extraction time, the r-protein in the supernatant can be recovered from the insoluble RNA by centrifugation. The disc electrophoresis patterns of all three preparations are virtually indistinguishable (Hardy et al., 1969), although Spahr (1962) reported different electrophoretic patterns with different isolation procedures. Usually the LiCl-urea procedure gives a higher yield of r-protein (>98%) but with more contamination of RNA than acetic acid extraction (yield 80-90%). Since virtually all of the r-protein can be extracted from the ribosome, when unfolded by urea, r-protein and RNA do not seem to be linked by covalent bonds.

Physicochemical characteristics of r-proteins from various ribosomal species will be discussed in detail elsewhere in this dissertation and only typical cases will be cited here. Waller and Harris (1961) determined the average molecular weight of E. coli 70S r-proteins to be 25,000 by amino acid end group, while Möller and Chrambach (1967) obtained 23,000 and 27,000 as weight average molecular weight ( $\bar{M}_w$ ) and z-average molecular weight ( $\bar{M}_z$ ) respectively for the same r-proteins. Setterfield et al. (1960), in their studies on r-proteins from pea seedlings, estimated the average isoelectric point to be about 8. Amino acid composition of all the r-proteins (unfractionated) from various species are very similar. In most cases they have a high content of both basic and acidic amino acids, and low content of sulfur-containing amino acids. Analytical results of  $\text{NH}_2$ -terminal groups are considerably different among different species, but a characteristic feature is that a large amount of alanine is their  $\text{NH}_2$ -end group.

An area which is entirely open with respect to plants and animals is the variation of r-protein patterns according to the life cycle. A few investigations have been done on microorganisms. Ennis and Lubin (1965) reported selective synthesis of r-protein during recovery from unbalanced growth in E. coli. Meselson et al. (1964) tried to investigate the conservation of the 30S and 50S ribosomes of E. coli during normal bacterial growth. They concluded that the 23S and 42S components were conserved during bacterial growth. In higher plant system, only physicochemical characterization of r-proteins from pea seedlings (Setterfield, et al., 1960; Birnstiel et al., 1964) and wheat embryo (Wolfe and Kay, 1968) have been reported. Therefore the present studies on r-protein



from pine seed ribosome is particularly interesting, because this plant is a representative of a fairly large group of commercially important gymnosperms, on which no previous work has been done. These studies may contribute fundamental knowledge about processes controlling germination of the seed. Ribosomal function may be involved in the germination procedure (Mans, 1967). The female gametophyte tissue of dormant pine seeds is known to contain inhibition factor of protein synthesis, which the embryo fraction lacks (Barnett, et al., 1970). The question arises: Are pine seed ribosomes in dormant state different from those in active germination and do pine seed ribosomes in dormancy have specific r-protein interacting with the inhibitor, thereby rendering dormant ribosomes inactive in protein synthesis?

The purpose of the present work is summarized as: (1) To develop technique for the investigation of the function of r-protein on the ribosomal structure; (2) To contribute to the understanding of ribosomal structure; (3) To provide fundamental knowledge about r-protein extracted from the seeds of Pinus lambertiana.

## II. REVIEW OF LITERATURE

### Characteristics of r-proteins

Waller and Harris (1961) were the first to demonstrate a high degree of electrophoretic heterogeneity of the r-protein extracted from E. coli ribosomes. This heterogeneity has been confirmed by others on all ribosomal preparations which have been studied (e.g. Table I). Waller (1964) subsequently demonstrated that the complicated electrophoretic pattern was independent of the urea concentration and was not affected by converting the protein to the S-sulfo derivative. The bands were reproducible upon rerunning. Thus, they confirmed that the observed electrophoretic heterogeneity was not the result of aggregation products due to protein-protein interaction, random disulfide bridge formation and/or protein-buffer interaction. After his report, similar work was reported by others (Traut, 1966; Möller and Chrambach, 1967). When Traut ran the gel electrophoresis with  $^{35}\text{S}$ -labeled r-proteins carried by excess unlabeled r-protein, the radioactivity appeared only in the expected regions and did not indicate any interactions with the excess carrier protein or any regeneration of additional regions of the pattern. He also mixed one radioactive subunit with a nonradioactive subunit to form 70S ribosomes. Ribosomal protein patterns from the hybrid ribosome and from the pure radioactive ribosome were the same, as their radioautographs were compared, indicating the absence of aggregation or bonding between proteins of the two subunits, either in the formation of 70S ribosome or in the preparation and electrophoresis of the protein mixture. By observing that samples of the derived and untreated controls produced

Table I Summary of the electrophoretic heterogeneity of r-protein species from various sources

Organism	S value	No. of Component bands	pH of electrophoresis	Electrophoresis material	Reference
<u>E. coli</u>	70	at least 20	5.6	starch	Waller and Harris (1961)
<u>E. coli</u>	70	about 24	3.5	15% polyacrylamide	Möller and Chrambach (1967)
<u>E. coli</u>	70	29	4.5	10% polyacrylamide	Low and Wool (1966)
<u>E. coli</u>	70	about 28	4.5	7.5% polyacrylamide	Leboy <u>et al.</u> (1964)
<u>E. coli</u>	70	25 or more	4.3	7.5% polyacrylamide	Traut (1966)
<u>E. coli</u>	50	34			Traub <u>et al.</u> (1969)
<u>E. coli</u>	30	19-22	4.5	10% polyacrylamide	Craven <u>et al.</u> (1969)
<u>E. coli</u>	30	approx. 20	4.5	10% polyacrylamide	Hardy <u>et al.</u> (1969)
<u>E. coli</u>	30	at least 17	4.3	7.5% polyacrylamide	Fogel and Sypherd (1968)
<u>E. coli</u>	30	20			Setterfield <u>et al.</u> (1960)
Heart (rat)	80	at least 24	4.5	10% polyacrylamide	Low and Wool (1966)
Skeletal muscle (rat)	80	at least 24	4.5	10% polyacrylamide	Low and Wool (1966)
Kidney (rat)	80	at least 24	4.5	10% polyacrylamide	Low and Wool (1966)
Reticulocyte (rabbit)	80	at least 24	4.5	10% polyacrylamide	Low and Wool (1966)
Liver (rat)	50	at least 17	4.6	polyacrylamide	Hamilton and Ruth (1967)
Liver (rat)	30	at least 12	4.6	polyacrylamide	Hamilton and Ruth (1967)
Paramecium	80	at least 34	4.5	7.5% polyacrylamide	Reisner <u>et al.</u> (1968)
Buds of pea seedlings	80	at least 16	4.2	starch	Setterfield (1960)
Wheat embryo	80	at least 26	4.5	10% polyacrylamide	Wolfe and Kay (1968)

the same pattern of bands in gel electrophoresis, Möller and Chrambach (1967) showed that the intermolecular disulfide bridge formation, deamidation of side-chain carboxyl groups of, phosphate or nucleotide bonding to, and/or carbamylation of, r-proteins are not responsible for the multiplicity of r-proteins from E. coli ribosomes. Delius and Traut (1969) observed unaltered protein patterns when ribosomes were prepared from cells washed and extracted in buffers containing the proteolytic enzyme inhibitor phenylmethyl-sulfonyl fluoride. However, definitive criteria for the heterogeneity have been obtained in several laboratories through the purification and characterization of the individual proteins, showing that each individual protein has a distinct primary structure (Kaltschmidt et al., 1967; Fogel and Sypherd, 1968; Moore et al., 1968; Hardy et al., 1967; Craven et al., 1969). From the 30S subunit of E. coli ribosomes Moore et al. (1968) obtained 13 proteins and Craven et al. (Kurland group) 20 proteins. The purity of the protein components was demonstrated by several criteria: electrophoretic and chromatographic homogeneity, homogeneity in acrylamide gels of several different porosities, physical homogeneity revealed by sedimentation equilibrium measurements and agreement between chemical molecular weight estimates and physical molecular weight estimates. All such data for individual r-proteins were unique and distinct. The complexity of NH<sub>2</sub>-terminal groups might be suggestive evidence for the heterogeneity of r-protein. By the end group analysis, the average molecular weight of r-protein was determined to be 25,000 for 70S E. coli ribosome (Waller and Harris, 1961), 12,700 for reticulocyte ribosome (Mathias and Williams, 1964), but the evidence and the nature of the size heterogeneity of

r-protein remained unspecified until Möller and Chrambach (1961) demonstrated obvious polydispersity of unfractionated E. coli r-proteins in sedimentation-diffusion and sedimentation equilibrium studies, with a  $\bar{M}_w$  of 23,000 and a  $\bar{M}_z$  of 27,000. Assuming a size distribution in which each class of molecules has the same concentration by weight, the range of molecular weights might vary between 10,000 and 40,000. A somewhat smaller spread (17,500 to 35,000) has been determined more directly via  $\text{NH}_2$ -terminal analysis of isolated r-protein fractions (Waller, 1964). Setterfield et al. (1960) reported polydispersity on ultracentrifugation of the acid soluble proteins of the ribosomes from buds of pea seedlings, with a mean sedimentation coefficient of about 0.9S and an average molecular weight of 18,000 by the Archibald technique. Hamilton and Ruth (1967) separated two of the protein components of rat liver ribosomes and determined the molecular weights by a sedimentation equilibrium technique to be 15,000 and 29,000. They also speculated that a range of 10,000 to 29,000 should include all the proteins of the large subunit of the rat liver ribosome. Wolfe and Kay (1968) reported the average molecular weight of r-proteins from wheat embryo as  $29,000 \pm 2,500$  and  $24,500 \pm 1,000$  by sedimentation-diffusion and osmometry, respectively. Moore et al. (1968) obtained 14,100 and 15,200 as  $\bar{M}_w$  of total 30S and 50S protein, respectively, but 70S protein had an apparent average molecular weight of 21,000.

Both the Kurland group (1970) and Traut et al. (1970), working separately to determine the mole fraction of each r-protein component in the E. coli 30S ribosomal subunit, observed that some proteins are present to the extent of one copy per ribosome but that there are some

proteins in amounts less than one copy per ribosome. Therefore the sum of molecular weights of all the 30S proteins exceeds the molecular weight of the unfractionated r-protein of the 30S subunit. However, their interpretation of the experimental data led them into quite opposite conclusions. The Kurland group proposed that the ribosome contains multiple copies of r-proteins, whereas a model of one copy of r-protein per ribosome was proposed by Traut et al. (also Moore et al.). The main factor which caused the two groups to draw opposite conclusions was the group of proteins which were not present in full molar amounts. The Kurland group obtained a group of proteins which were present in amounts less than 0.7 copies per ribosome (in addition to 0.8 and 1.2 copies, which were agreed upon by both groups), and they estimated that such deviations from one copy was well beyond their experimental error. On the other hand, Traut et al. tried to explain such deviations from stoichiometric amounts in several ways: smaller deviations may be due to experimental error and can be neglected; the proteins with considerable deviations could be non-ribosomal contaminants, not required for ribosome structure or activity; the proteins could be r-proteins which were loosely bound and lost during ribosome preparation; they could be proteins which are needed for ribosome function during only part of the protein synthesis cycle, acting catalytically as "nonribosomal" factors. However, both groups observed some very obscure bands of protein components and they were not sure about partial loss of r-proteins or contamination with extraneous proteins in their experiments.

Literatures of other physicochemical properties of r-proteins, such as amino acid composition, NH<sub>2</sub>-terminal group, sedimentation and

spectrophotometric patterns, are reviewed elsewhere in this dissertation.

#### Function of r-protein in ribosome structure

Several workers (Spirin, 1964; Hill, 1969) noticed the concomitance of ribosomal translocation with active transport, which is probably due to an allosteric protein with two discrete conformational states. The occurrence of such changes in the conformation of the ribosome during its function is suggested by a number of phenomena, e.g. the translocase reaction (Heintz et al., 1969), the movement of the ribosome along the messenger RNA, and the dissociation of the ribosome, which has been shown to involve a conformational change in the subunits (Page et al., 1967). Sherman et al. (1969) suggested that (dihydro)streptomycin exerts its action of miscoding on the ribosome by a deformation of ribosomal structure through the mediation of specific r-proteins. The importance of the r-protein in the maintenance of the structure of r-RNA has been stated by a number of workers e.g. Spirin (1964). Arnott et al. (1968) reported that RNA can exist in three distinct transition forms and speculated that changes in the internal arrangements of RNA helices might be associated with the binding and successful recognition of an aminoacyl t-RNA, the movement of t-RNA and of m-RNA, from the amino acid to the peptidyl position, and finally the removal of the t-RNA from the binding site so that the cycle can be repeated. Streptomycin may affect these processes through r-protein, which is important for the RNA conformation.

The ribosome has some loosely bound proteins and some proteins which are present in less than stoichiometric amounts. Whether or not these two kinds of proteins are identical is not known. They have been neither characterized nor identified. However, these may be the key proteins for the solution of questions regarding the presence of one copy or multiple copies of r-proteins and homogeneity or heterogeneity of ribosomes. One might speculate that these proteins may affect the state of the ribosome irrespective of the origin of the proteins. The Kurland group (1970) proposed a steady-state model and a static model in their studies on the 30S ribosomal subunit of E. coli. This was based on those proteins which were present in amounts less than 0.7 copies per ribosome and were called fractional proteins. The Kurland group proposed heterogeneity of ribosomes on the basis of these proteins. However, recently Hill et al. (1969) confirmed the molecular weights of both of the 30S and 50S subunit of E. coli ribosomes by the rather stringent tests of the sedimentation equilibrium method and concluded that 30S subunit was homogeneous. However, we should notice that he washed the 70S ribosomes overnight in a buffer containing 0.5M  $\text{NH}_4\text{Cl}$  prior to the molecular weight estimation, and the degree of detachment or contamination of r-protein might be dependent upon the washing method.



### III. MATERIALS AND METHODS

#### Ribosome preparation

Dry seeds of Pinus lambertiana were provided by Forest Seeds of California, Davis, California, and were stored in the cold until used. All chemicals used were reagent grade.

The procedures of the ribosome preparation are outlined in Figure 1. Fifty grams of pine seeds were washed thoroughly with tap water by shaking for one hour, washed three times with distilled water and once more with quartz redistilled water. The washed seeds were presoaked in the cold with quartz redistilled water overnight. All the following steps were done at 4°C. The presoaked seeds were mixed with 80 ml of 0.0063M MgCl<sub>2</sub>, 0.063M Tris-HCl (pH 8.3), 0.025M KCl, 0.0075M 2-mercaptoethanol, and 0.5M sucrose, and 20 ml of 2.5% aqueous sodium desoxycholate immediately before homogenization. The resulting 100 ml homogenization medium contained 0.005M MgCl<sub>2</sub>, 0.005M Tris HCl (pH 8.3), 0.02M KCl, 0.006M 2-mercaptoethanol, 0.4M sucrose, and 0.5% sodium desoxycholate. The pine seeds in the medium were homogenized for two minutes in a Sorvall Omni-mixer. The homogenate was squeezed through cheese cloth and centrifuged for 30 minutes at 27,000 x g and the semisolid lipid layer and the precipitate were discarded. The supernatant was centrifuged for 3 hours at 105,000 x g. The 105,000 x g pellet was suspended in 40 ml of 0.005M MgCl<sub>2</sub>, 0.02M KCl, 0.006M 2-mercaptoethanol, 0.025M Tris-HCl (pH 7.8) (this buffer system will be referred as buffer B), centrifuged for 30 minutes at 27,000 x g and the supernatant was again centrifuged for 2 hours at 105,000 x g. The resulting pellet was called B<sub>1</sub>-ribosome and the supernatant B<sub>1</sub>-supernatant. The B<sub>1</sub>-ribosome pellet was suspended in

Figure 1. Schematic representation of ribosome preparation procedure.

50 g. of dry Seeds

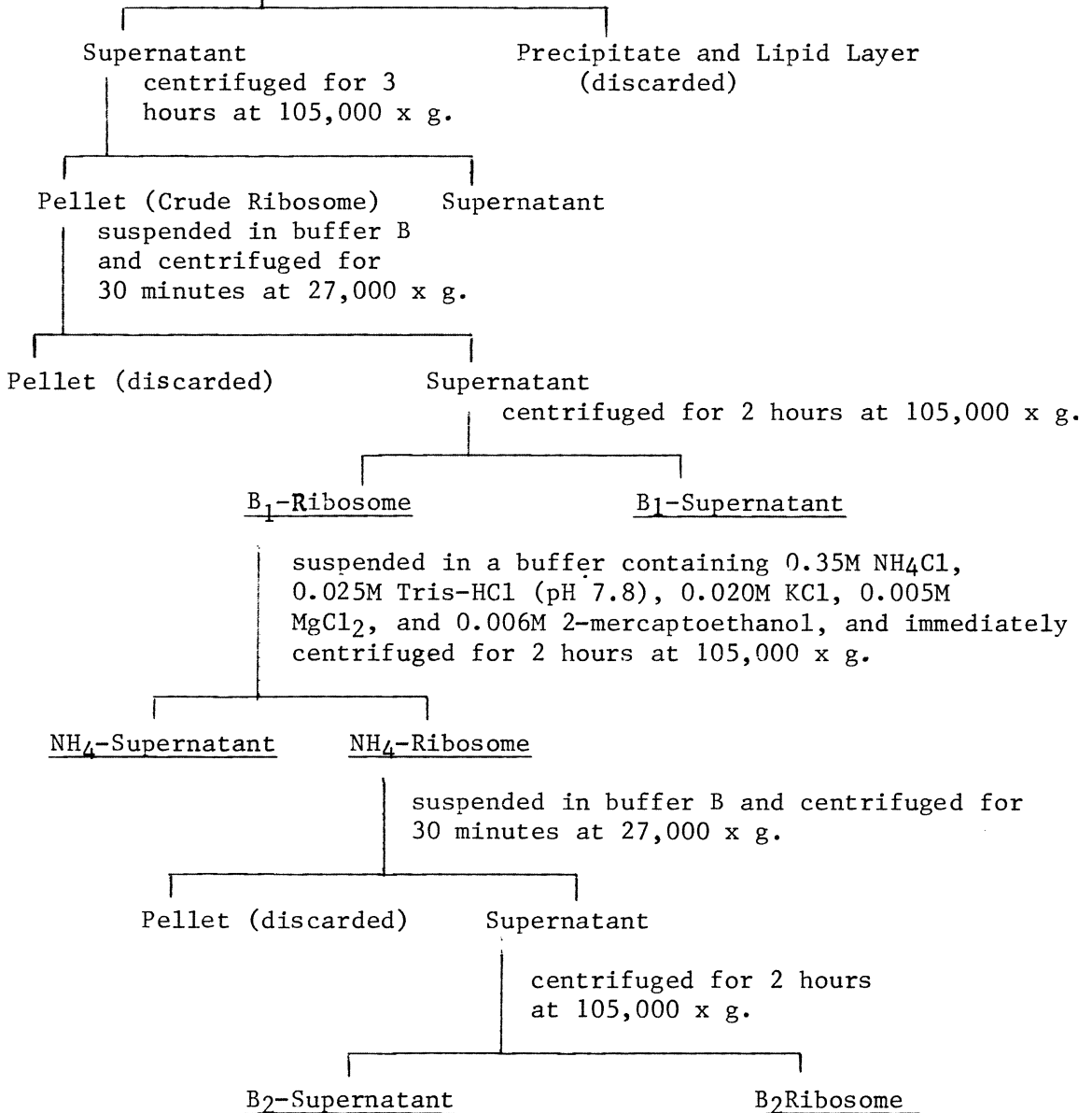
washed and soaked in cold distilled water overnight at 4°C.

Presoaked Seeds

homogenized in 100 ml. of homogenization buffer containing 0.4M sucrose, 0.005M MgCl<sub>2</sub>, 0.05M Tris-HCl (pH 8.3), 0.02M KCl, 0.006M 2-mercaptoethanol, and 0.5% DOC, for 2 minutes in Sorvall Omni Mixer.

Homogenate

squeezed through cheese cloth and the filtrate was centrifuged for 30 minutes at 27,000 x g.



20 ml of 0.35M  $\text{NH}_4\text{Cl}$ , 0.025M Tris HCl (pH 7.8), 0.020M KCl, 0.005M  $\text{MgCl}_2$ , 0.006M 2-mercaptoethanol, and immediately the high spin centrifugation was repeated. Resulting ribosome pellet was designated as  $\text{NH}_4$ -ribosome and the supernatant as  $\text{NH}_4$ -supernatant. After resuspension in 20 ml of buffer B, the high and low spin centrifugations were repeated. The pellet and supernatant were called  $\text{B}_2$ -ribosome and  $\text{B}_2$ -supernatant, respectively. Frequently this  $\text{B}_2$ -ribosome will also be called 80S ribosome or simply ribosome in this dissertation.

#### Dissociation and preparation of ribosomal subunits

Two ml of a 1% ribosome suspension in  $6 \times 10^{-4}\text{M}$   $\text{MgCl}_2$ , 0.025M Tris-HCl (pH 7.8), and 0.020M KCl (dissociation buffer) were applied onto a 8-32% (w/v) linear sucrose gradient in the dissociation buffer. The sucrose gradient was made in cellulose nitrate tube by the procedure of Brakke (1960). Centrifugation was done in the SW-27 rotor of the Spinco Model L-2 65B ultracentrifuge at 23,000 rpm for 9 hours. The gradients were analyzed and the subunits were collected using an ISCO Gradient Fractionator, monitored at 254 nm. The collected subunit fractions were diluted with an equivalent volume of dissociation buffer and pelleted by centrifugation at 55,000 rpm for 3 hours in the 65 rotor of the Model L-2 65B ultracentrifuge. The supernatant was decanted and the pelleted subunits were gently rinsed with the dissociation buffer. All of these studies were performed at  $4.0^\circ$ .

#### Preparation of ribosomal proteins

Ribosomal protein was extracted from each ribosomal species, i.e. 80S, 40S and 60S, which had been prepared by the aforementioned procedures, either by acetic acid extraction by the method of Waller

and Harris (1961) or by a modification of LiCl-urea method of Leboy et al. (1964). Acetic acid method: To a cold solution of ribosomes (1-3%) by weight in buffer B, two volumes of concentrated acetic acid (99.9%) were added very slowly while keeping the ribosome solution agitated with a tiny magnetic stirring bar. After stirring gently in the cold for 45 minutes, the RNA precipitate was collected by centrifugation at 27,000 x g. The supernatant solution was dialyzed against distilled water in the cold and lyophilized. LiCl-urea method: A solution of 6M LiCl and 8M urea was added to an equal volume of 1-3% suspension of ribosomes in buffer B. The solution was mixed and held 24 hours in the cold, following which the precipitated RNA was removed by centrifugation at 100,000 x g for 20 minutes. The supernatant was dialyzed against 0.01M HCl for 48 hours in the cold. The dialyzed protein solution was centrifuged at 13,000 x g for 2 hours and lyophilized.

#### General procedures

Dialysis: Dialysis was carried out in the cold. The dialysis tubings were from Viking Company or Union Carbide Corporation.

Analytical Ultracentrifuge: The Spinco Model E Ultracentrifuge equipped with schlieren optics was used for all sedimentation experiments. The centrifuge was run at 3-7°C for ribosome species and at 8-10°C for r-protein species. The r-protein solution was centrifuged in a capillary type synthetic boundary cell. Observed sedimentation coefficient were corrected to the viscosity and density of water at 20°C ( $s_{20,w}$ ).

Ultraviolet spectrophotometry: Ultraviolet absorption spectra of samples were scanned with Unicam SP.800 A Ultraviolet Spectrophotometer.

The temperature of cell holder was kept at the desired temperature by circulating water using the Brinkman Ultra-Thermostat. For measuring absorbance, Beckman DU Spectrophotometer or Unicam SP.800 A Ultraviolet Spectrophotometer was used.

#### Disc gel electrophoresis

TEMED( $N_1N_1N_1N_1'$ -tetramethylethylenediamine), acrylamide, Bis ( $N_1N_1'$ -methylenebisacrylamide), riboflavin, aniline blue black, and column coat were purchased from Canal Industrial Corporation.  $\beta$ -Alanine was obtained from Eastman Organic Chemical. Urea(extra pure) was purchased from Mann Research Laboratories, Schwarz BioResearch. Disc gel electrophoresis of all species of r-proteins, i.e. 80S r-proteins, 60S r-proteins, 40S r-proteins, and 80S r-proteins extracted from the dansylated ribosome (80S DNS-r-protein) was carried out in the pH 4.5 system of 15% polyacrylamide gels containing 8M urea, described by Reisfield et al. (1962) and by Leboy et al. (1964) with slight modifications, as described below. Forty  $\mu$ g r-proteins were loaded per column. The electrophoretic run was carried out at 4° for 1 hour and 35 minutes. A constant current of 4mA per tube was applied with the anode in the upper chamber. The Canalco, Model 6 System was used.

#### Isoelectric focusing in polyacrylamide gel

Ampholine of pH range 3-10 and dry content 40% (w/v) was from LKB Produkter, and ethylenediamine(anhydrous) was from Fisher Scientific Company. Other chemicals used were those described in disc gel electrophoresis. Stock solutions were made as follows:

Solution A:	acrylamide	6.25 g
	Bis	0.25 g
	urea	24 g, water to total of 50 ml.

Solution B: TEMED	0.3 ml
urea	24 g., water to total of 50 ml
Solution C: ampholine	5 ml
urea	24 g, water to total of 50 ml
Solution D: riboflavin	2 mg
urea	24 g., water to total of 50 ml
Cathode solution:	5% (v/v) ethylenediamine
Anode solution:	5% (v/v) phosphoric acid

Just prior to use, the working solution was prepared by mixing: 4A+1B+4C+1D. The resulting working solution contains 5% acrylamide, 0.06% TEMED, 0.4% riboflavin, 1.6% ampholine, and 0.2% Bis. Eighty  $\mu$ g r-protein was dissolved directly in 2.5 ml of the above mixed working solution. This protein containing working solution was introduced slowly into glass tubes which were 12.5 cm in length and 0.5 cm in internal diameter. The lower (anode) end was inserted into a cap provided by Canalco for disc gel electrophoresis up to a mark etched on the tube at distance 11.5 cm from the lower (anode) end. These glass tubes had been coated with column solution before the use. A small amount of water (3-4 mm deep) was added to the column to form a layer over the gel. Gel columns were placed about 30 cm from the photopolymerizing light source. After 10 minutes, when polymerization had started as evidenced by the appearance of opalescence, the light source was moved close to the columns for an additional 15 minutes. The water layer was drained from the column after polymerization. The lower (anode) end and upper (cathode) end could be distinguished by the difference of the shape of meniscus. The upper end, which had been layered with water during polymerization, was more flat than the lower end, which had been inserted into a rubber cap. The upper (cathode) end can also be identified by the protein band which

the lower (anode) end lacked, when the electrophoresis was completed. The column tubes were inserted into the apparatus and the upper air spaces were filled with the upper electrode solution (5% ethylenediamine). Isoelectrofocusing was performed in the assembly used for disc gel electrophoresis (Canalco, 6 system) in the cold (4°C). The upper bath was filled with 200 ml of 5% ethylenediamine solution and the lower bath with 750 ml of 5% phosphoric acid. A constant voltage of 140 volt and an initial current of 1.2 mA per gel column was applied using Spinco Duostat of Beckman Instruments. The current steadily fell to constant current of about 0.1 mA per column after about 2 hours. Electrophoresis was run for 18 hours. When the electrofocusing was completed the gel was removed from the glass tubes and put in 12% TCA solution for a few hours to precipitate the protein bands in the gel and to allow ampholine carrier ampholites to diffuse out. The precipitated protein bands could be seen clearly at the cathode end. This was a good marker for distinguishing the cathode and the anode ends of the column. The gel column was washed with 6% TCA solution for about twelve hours with several changes of the 6% TCA solution. After washing, the column was stained overnight with 0.5% aniline black dye in 7% acetic acid. Destaining was performed electrophoretically in 7% acetic acid with Beckman Spinco Duostat as described for disc electrophoresis. The pH range of the gel column was determined by slicing a blank gel column (without protein) at uniform intervals of 3mm. The sliced gel was soaked in one ml of distilled water for several hours, and the pH was measured at 25° with a microelectrode.



### Sequential release of proteins from 80S ribosomes

Particles ( $B_1$ -ribosome,  $NH_4$ -ribosome, and  $B_2$ -ribosome) obtained at each stage of ribosome preparation described earlier were treated with 6M LiCl-8M urea by the same procedure used in the preparation of r-protein by the LiCl-urea method, and the proteins extracted were processed according to Atsmon et al. (1966) and Spitnik-Elson and Atsmon (1969). The proteins were dialyzed against cold 0.01N HCl for 48 hours, or until the solution was almost clear, centrifuged 2 hours in the cold at 13,000 x g and lyophilized. The same course of dialysis, centrifugation and lyophilization was employed on each supernatant fraction ( $B_1$ -supernatant,  $NH_4$ -supernatant, and  $B_2$ -supernatant).

### General dansylation procedure of the ribosome

DNS-Cl (1-dimethylaminonaphthalene-5-sulfonyl chloride) dispersed on celite, colorimetric assay 11%, was purchased from Calbiochem and methyl- $C^{14}$ -dansyl chloride in acetone, specific activity 45 mc/mm, was from Schwarz Bioresearch Inc. To 5 ml of 0.6% ribosome, in a medium containing 0.025M cacodylate (pH 8.8), 0.02M KCl and  $MgCl_2$  at the desired level, were added 0.1 grams of DNS-Cl-celite, which corresponds to 4 $\mu$  moles of DNS-Cl, and stirred gently for 2.5 hours (except for time course study) in the cold. The reaction mixture was then centrifuged three times at 12,000 x g for 10 minutes to eliminate DNS-celite residues. The precipitate was discarded each time. The supernatant was dialyzed overnight in the cold versus a medium composed of 0.025M Tris-HCl (pH 7.8), 0.02M KCl with  $MgCl_2$  at the appropriate level. In the study of the effect of dansylation on ribosomes, the system contained celite

instead of DNS-Cl-celite but the other components were the same as in the dansylation system, was used as a control system.

#### Time course of the dansylation of ribosomes

The time course of the dansylation of ribosomes was estimated by measuring the uptake of methyl- $C^{14}$ -DNS-Cl loaded on DNS-Cl-celite. A solution of 10mc (0.22 mmoles) methyl- $C^{14}$ -DNS-Cl (45 mc/mm) in 0.4 ml acetone was added to 0.7 g. DNS-Cl-celite (0.285mmoles of DNS-Cl). Then the DNS-Cl-celite loaded with methyl- $C^{14}$ -DNS-Cl was added to the ribosome solution of 21 mg in 35 ml buffer B, and the dansylation reaction was carried out as described in the above general procedure. One ml portions of the reaction mixture were taken at intervals and centrifuged for 5 minutes at 1,000 x g. To the supernatant was added 1 ml of 10% TCA. The precipitate was washed twice with 2 ml of 5% TCA and finally once with 2 ml of acetone-10% TCA (1:9v/v). The washed precipitates were dissolved by the addition of small amount (0.3 ml per tube) of 98% formic acid, transferred onto aluminum planchets, air dried, and the radioactivity was counted by Nuclear Chicago gas flow counter. Correction for self absorption was not done.

#### Comparison of the degree of dansylation of 1M LiCl split free protein and protein of particulate fraction of 80S DNS-ribosome.

The ribosome was dansylated with DNS-celite loaded with methyl- $C^{14}$ -DNS-Cl in the medium containing  $5 \times 10^{-3}M$   $MgCl_2$ , 0.025M cacodylate (pH 8.8) and 0.020M KCl as described in the general dansylation procedure. The unreacted DNS-Cl-celite residues were eliminated by centrifugation at 1,000 x g, and the dansylated ribosomes were dialyzed against a medium composed of  $1.5 \times 10^{-3}M$   $MgCl_2$ , 0.025M-Tris (pH 7.8) and 0.020M KCl as

described in the general dansylation procedure. The  $C^{14}$ -radioactive DNS-ribosomes suspended in the above dialysate buffer were mixed with an equal volume of medium containing 2M LiCl,  $1.5 \times 10^{-3}$ M  $MgCl_2$ , 0.025M-Tris-HCl (pH 7.8) and 0.020M KCl for 30 minutes in the cold. Then the reaction mixture was centrifuged at  $150,000 \times g$  for 5 hours to isolate the free protein fraction (supernatant) and particulate fraction (pellet). Protein in the particulate fraction was separated from RNA by the LiCl-urea method. The final volumes of each protein fraction (split free and particulate protein) were adjusted to 10 ml with buffer B. Portions of 2 ml were taken, precipitated with 2 ml 10% TCA, washed twice with 2 ml 5% TCA, and finally with 2 ml acetone-10% TCA (1:9, v/v). The radioactivity of washed DNS-r-protein precipitates was measured according to the method used in the time course of the dansylation of ribosomes. The protein concentration of the split free protein and particulate protein were determined according to the method of Lowry et al. (1951). The comparison of the degree of dansylation of the two protein fractions was done using the radioactivity data and protein analysis data.

#### Infra-red spectrophotometry

Pellets of ribosomes ( $B_1$ -ribosome,  $NH_4$ -ribosome, and  $B_2$ -ribosome) and r-protein were lyophilized and pulverized with a spatula before use. Potassium bromide of reagent grade, A.C.S. Crystals was from Matheson Coleman and Bell. Either 1.5 mg or 3 mg of the sample were mixed with 100 mg of powdered KBr. A four mg portion of the mixture was transferred to Beckman Micro-Pellet Die and pressed into transparent KBr pellet with a total force on the ram of 700 pounds using Carver Laboratory Press of Fred S. Carver, Inc. All spectra were scanned with Beckman IR-5A Infrared

Spectrophotometer equipped with an attenuator in the reference beam path and with a beam condenser in the sample beam path.

Effect of ribonuclease on the ribosomes of 80S, 60S, 40S or 80S DNS-ribosome

A 0.03 ml of 0.5% sample in buffer B was suspended in 3 ml of medium (preincubated to 30°) containing 0.025M Tris-HCl (pH 7.8), 0.020M KCl, and MgCl<sub>2</sub> at desired concentration, which was held in the spectrophotometer cuvette and allowed to stand at 30° for 2 minutes. On addition of 30 mg of ribonuclease (beef pancreatic purchased from Worthington Biochemical Corporation) dissolved in 0.03 ml of quartz distilled water, the absorbance at 260 nm was scanned at one minute intervals until plateau was reached. The reaction temperature was kept at 30°. The zero time absorbance was 0.4-0.6 at 260 nm.

Temperature-absorbance profile at 260 nm

Ribosome samples were suspended in preincubated buffer containing  $1.5 \times 10^{-3}$  M MgCl<sub>2</sub>, 0.025M Tris-HCl (pH 7.8) and 0.020M KCl. The initial absorbance was 0.4-0.7 at 260 nm. The temperature-absorbance profile was scanned with Gilford 2400 Spectrophotometer. The temperature was corrected with E. coli B RNA, whose T<sub>m</sub> was 90.5° in 0.15 M NaCl, 0.015M Na<sub>3</sub>citrate. The rate of heating was 1.63° per minute. Correction for thermal expansion of water was not applied.

Determination of molecular weight of r-protein by sedimentation equilibrium analysis

The molecular weight of r-protein was determined by the method of Yphantis solution meniscus-depletion and sedimentation equilibrium (Yphantis, 1964). The r-protein sample solution was prepared at a concentration of 0.1% at pH 4.7 in 8M urea, 0.15M KCl, 0.05M acetic acid,

0.02M sodium acetate, and 0.005M DTT (dithiothreitol). Correction was not made to zero concentration. A partial specific volume of 0.73 was calculated from the amino acid analysis.

### Chemical analysis

Amino-terminal amino acid determination: Dansylation and hydrolysis of the DNS-r-protein was performed according to Gros and Labouesse (1969) with slight modifications, and high voltage electrophoresis was used as described by Gray (1967). To one mg of r-protein in 0.5 ml of a medium containing 8M urea, 0.4M phosphate buffer (pH 8.8, potassium salt) and 0.25 ml of dimethylformamide, was added 0.2 ml of 2M DNS-Cl dissolved in acetonitrile. The reaction mixture was allowed to stand, with frequent agitation, for 30 minutes at room temperature, and then 10 ml of 10% TCA was added to precipitate down the dansylated proteins. The mixture was centrifuged for 15 minutes at 1.000 x g. The precipitated protein was washed twice with 10 ml of 1N HCl. Then, a half ml of 6N HCl was added to the precipitate tube, and the air space of the tube was replaced by nitrogen gas. The tube was drawn out in a fine oxygen-gas flame, cooled in a stream of air, and sealed quickly in the flame. The sealed tube was heated in an autoclave at 110°C for 4 hours and 18 hours. The hydrolysate was dried in vacuo, using the Rotary Evapo-Mix of Buchler Instruments. For any methionine and cysteine end groups, preliminary performic acid oxidation according to Fraenkel-Conrat et al. (1955) was done on r-protein as follows: A mixture of one volume of 30% H<sub>2</sub>O<sub>2</sub> and 9 volumes of 88% formic acid was allowed to stand for one hour at room temperature. Twenty five ml of the performic acid reagent, precooled to 0°, was added to 0.1 gram of r-protein, and kept at 0° for

4 hours. The solution was then evaporated to dryness using the Rotary Evapo-Mix of Buchler. The dried residue was used for  $\text{NH}_2$ -terminal group analysis in the usual way described above. The dansylated, hydrolyzed, and dried sample was dissolved in a mixture of acetone-1N HCl (9:1, v/v) and applied on an appropriate paper (23 cm width and 60 cm length) along with standard DNS-amino acids. Standard DNS-amino acids were prepared by mixing one ml of amino acid (6.5 mmoles in 0.1M  $\text{NaHCO}_3$ ) and one ml of DNS-Cl in acetone (6 mg/ml) and allowing the mixture to stand overnight. Sodium bicarbonate ( $\text{NaHCO}_3$ ) was precipitated by the addition of 8 ml acetone and the supernatant was ready for the electrophoresis. After applying the sample and standards, the paper was wetted by spraying with electrophoresis solvent. The electrophoresis plate was precooled and maintained between  $10^\circ$  and  $15^\circ$  during the run. Three systems used as electrophoresis solvents were: (1) pH 4.4 (0.8% acetic acid-0.4% pyridine). Two hours of run at 80 v/cm using Whatmann No. 3mm as paper; (2) pH 12.7 (0.1M  $\text{Na}_3\text{PO}_4$ -0.1M NaOH) for 2 hours at 20v/cm using Whatmann No. 52 paper. Origin was placed near the cathode; (3) pH 1.9 (formic acid, 8% v/v) run for one hour at 50 v/cm using Whatmann No. 1 paper. On completion of the electrophoresis, the paper was dried at room temperature with a hair dryer, and the developed dansyl amino acid (DNS-amino acid) spots of fluorescence were located with an ultraviolet lamp (Mineralight, Model RS 1, Ultraviolet Products, Inc.).

Amino acid analysis: A 5 mg of the protein sample was hydrolyzed for 36 hours in 5 ml 6N HCl at  $110^\circ\text{C}$  under nitrogen gas in sealed tubes. The hydrolysate was dried under vacuum using Rotary Evapo-Mix,

Buchler Instruments. Amino acid analysis was carried out with a Beckman Amino Acid Analyzer, Model 120B. Correction for the destruction of amino acids during hydrolysis was not made.

Determination of protein and RNA: Thoroughly washed ribosomes were suspended in 2 ml of distilled water and 10 ml of 1M KOH and 0.05M ethylenediaminetetraacetate (EDTA, potassium salt) were added. Then, the solution was incubated at 37° for 5 hours with agitation. Five ml of 30% TCA solution containing 2M HCl were added to the dissolved ribosome solution and allowed to stand for 15 minutes. The precipitate formed was collected by centrifugation and washed once with 5 ml of 10% TCA solution. The supernatant and wash solution was combined, made to 25 ml by the addition of distilled water, and used for RNA analysis. Precipitates were dissolved by the addition of 5 ml of 1N NaOH and used for protein analysis. RNA determination was performed by the cupric ion catalyzed orcinol reaction according to Lin and Schjeide (1969). Standard RNA was prepared by incubating 100 mg of yeast RNA (from Pabst Laboratories) in 25 ml of 1N KOH and then neutralizing with 1N HCl and diluting with distilled water to 100 ml. Orcinol (reagent grade from Fisher Scientific Company) was recrystallized twice from benzene. Bovine serum albumin, from nutritional Biochemicals Corporation, was used as the standard of protein determination according to the method of Lowry et al. (1951).

#### IV. RESULTS

##### Reaction of DNS-Cl with the ribosome

DNS-ribosome was separated into the protein and the RNA moieties by LiCl-urea extraction. The protein fraction was dialyzed, centrifuged, and lyophilized as stated in the section of the preparation of r-protein. The RNA fraction (pellet) was suspended and washed with a medium containing an equal volume of buffer B and of the mixture of 6M LiCl-8M urea and then dissolved in a buffer containing 0.1M KCl, 0.1M Tris-HCl (pH 9) and 0.01M ethylenediaminetetraacetate, dialyzed against the same buffer, and lyophilized. When the fluorescence of both protein and RNA was checked the protein had characteristic fluorescence both before and after lyophilization, while the RNA did not.

##### Kinetics of DNS-Cl uptake by the ribosome

Figure 2 shows the uptake of DNS-Cl by the ribosomes at a  $\text{MgCl}_2$  concentration level of  $5 \times 10^{-3}\text{M}$ . The time indicated is that at which samples were removed from the reaction mixture. In the calculation of the uptake of DNS-Cl as a function of time, it was assumed that the uptake of the non-radioactive carrier DNS-Cl was the same as that of the methyl- $\text{C}^{14}$ -DNS-Cl. The uptake increased linearly and reached a plateau after 2.5 hours, with about 45  $\mu\text{m}$  moles of DNS-Cl bound per mg of ribosomes. This corresponds to 180 molecules of DNS-Cl per ribosome particle, assuming the molecular weight of ribosome to be  $4 \times 10^6$  (Petermann, 1964).

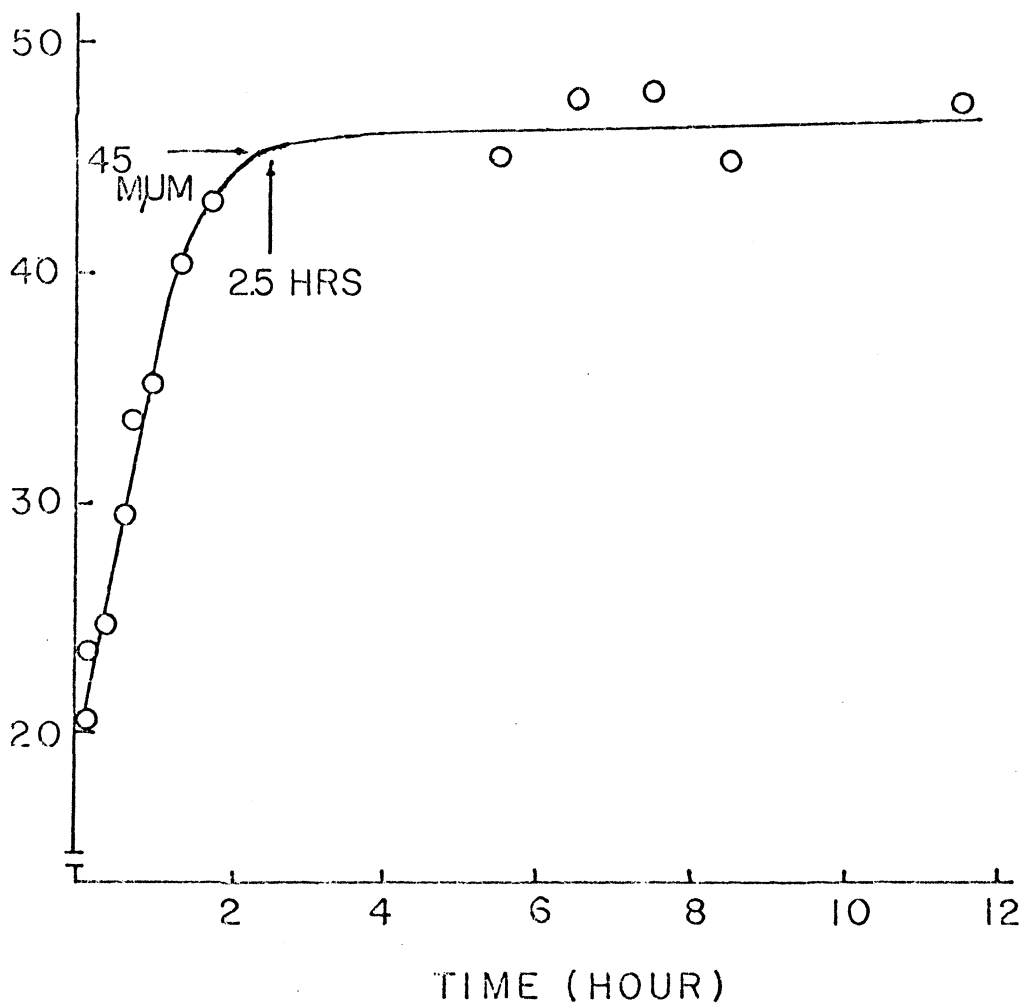
##### Effect of the dansylation on the sedimentation patterns of ribosomes

Figure 6, 3, and 8 show the effect of dansylation of ribosomes at  $\text{MgCl}_2$  concentration levels of  $5 \times 10^{-3}\text{M}$ ,  $1.5 \times 10^{-3}\text{M}$ , and  $6 \times 10^{-4}\text{M}$  on their



Figure 2. Time course of the dansylation of pine seed ribosomes. To twenty one mg ribosomes in 35 ml of a medium containing 0.025M cacodylate (pH 8.8),  $5 \times 10^{-3}$ M  $MgCl_2$ , 0.020M KCl, were added 0.7 g DNS-celite carrying 10 mc methyl- $C^{14}$ -DNS-Cl (45 mc/mmole) and the reaction was carried out in the cold ( $4^{\circ}$ ). Portions were removed and counted as described in the materials and methods. The data show the number of  $\mu$  moles DNS-Cl bound per mg of ribosomes. The amount of ribosomes was computed from the absorbance data at 260 nm and 280 nm, and assuming the protein content of ribosome to be 30%.

DNS-Cl (MUMOLE) PER MG RIBOSOMES



sedimentation patterns at the same  $\text{MgCl}_2$  concentration levels as those of dansylation. At all  $\text{MgCl}_2$  concentration levels tested, there is not any difference in the sedimentation patterns between the dansylated and control(not dansylated).

#### Effect of dansylation on dissociation of ribosomes

When ribosomes dansylated at high  $\text{MgCl}_2$  concentration ( $5 \times 10^{-3}\text{M}$ ) were dialyzed against a buffer of low  $\text{MgCl}_2$  concentration ( $6 \times 10^{-4}\text{M}$ ), the dissociation pattern was same as that of control (not dansylated) (Figure 5). Thus dansylation has no effect on the dissociation of ribosomes.

#### Effect of dansylation on reassociation of ribosomes

Figure 7 shows the effect of dansylation of dissociated ribosomes (at  $6 \times 10^{-4}\text{M}$   $\text{MgCl}_2$  concentration) on their reassociation at high  $\text{MgCl}_2$  concentration ( $5 \times 10^{-3}\text{M}$   $\text{MgCl}_2$ ). Figure 4 shows the effect of dansylation of ribosomes at  $1.5 \times 10^{-3}\text{M}$   $\text{MgCl}_2$  on their sedimentation pattern at  $5 \times 10^{-3}\text{M}$   $\text{MgCl}_2$ . The reassociation patterns of the dansylated ribosomes are same as their corresponding controls (not dansylated) and the dansylation has no effect on the reassociation of dansylated ribosomes.

#### Effect of the $\text{MgCl}_2$ concentration of dansylation on the sedimentation patterns of ribosomes

When ribosomes dansylated at different  $\text{MgCl}_2$  concentrations were dialyzed against a buffer of same  $\text{MgCl}_2$  concentration, their sedimentation patterns were the same: Ribosomes dansylated at the  $\text{MgCl}_2$  concentration of  $1.5 \times 10^{-3}\text{M}$  (Figure 4),  $5 \times 10^{-3}\text{M}$  (Figure 5), or  $6 \times 10^{-4}\text{M}$  (Figure 7) had same sedimentation patterns when they were dialyzed at  $5 \times 10^{-3}\text{M}$   $\text{MgCl}_2$  concentration; ribosomes dansylated at the  $\text{MgCl}_2$  concentration of  $6 \times 10^{-4}\text{M}$

Figure 3. Analytical ultracentrifuge pattern of ribosomes (6mg/ml) dansylated at  $1.5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$  concentration level and dialyzed vs. a buffer containing  $1.5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$ , 0.025M Tris-HCl (pH 7.8), 0.02M KCl, and 0.006M 2-mercaptoethanol. Schlieren patterns of control and dansylated ribosomes were taken at a phase angle of  $50^\circ$  and 12 minutes after the centrifuge reached 39,460 rpm at  $3.0^\circ$ . Sedimentation was from right to left. Numbers in figure are sedimentation coefficients in svedberg unit ( $S_{20,w}$ ).

Top: DNS-ribosome

Bottom: control

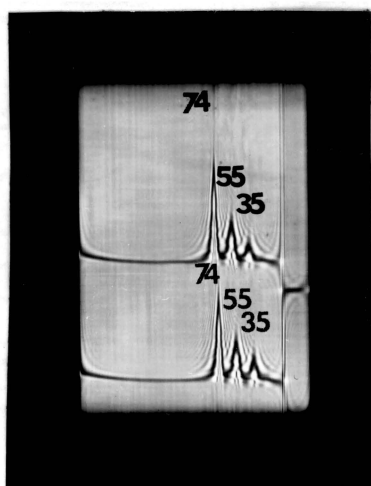


Figure 4. Analytical ultracentrifuge pattern of ribosomes (6mg/ml) dansylated at  $1.5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$  concentration level and dialyzed vs. a buffer containing  $5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$ , 0.025M Tris-HCl (pH 7.8), 0.02M KCl, and 0.006M 2-mercaptoethanol. Schlieren patterns of control and dansylated ribosomes were taken at a phase angle of  $50^\circ$  and 12 minutes after the centrifuge reached 39,460 rpm at  $5.5^\circ$ . Sedimentation was from right to left. Numbers in figure are sedimentation coefficients in svedberg unit ( $S_{20,w}$ ).

Top: DNS-ribosome

Bottom: control

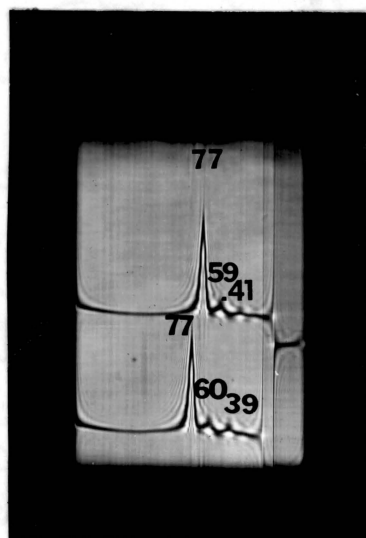


Figure 5. Analytical ultracentrifuge pattern of ribosomes (6mg/ml) dansylated at  $5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$  concentration level and dialyzed vs. a buffer containing  $6 \times 10^{-4} \text{M}$   $\text{MgCl}_2$ , 0.025M Tris-HCl (pH 7.8), 0.020M KCl and 0.006M 2-mercaptoethanol. Schlieren patterns of control and dansylated ribosomes were taken at a phase angle of  $50^\circ$  and 12 minutes after the centrifuge reached 39,460 rpm at  $4.4^\circ$ . Sedimentation was from right to left. Numbers in figure are sedimentation coefficients in svedberg unit ( $S_{20,w}$ ).

Top: DNS-ribosome

Bottom: control



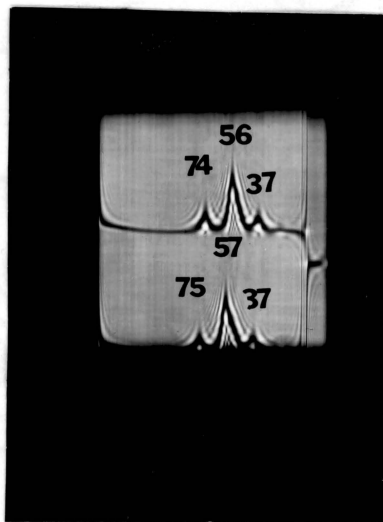


Figure 6. Analytical ultracentrifuge pattern of ribosomes (6mg/ml) dansylated at  $5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$  concentration level and dialyzed vs. a buffer containing  $5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$ , 0.025M Tris-HCl (pH 7.8) 0.020M KCl and 0.006M 2-mercaptoethanol. Schlieren patterns of control and dansylated ribosomes were taken at a phase angle of  $50^\circ$  and 14 minutes after the centrifuge reached 39,460 rpm at  $7.0^\circ$ . Sedimentation was from right to left. Numbers in figure are sedimentation coefficients in svedberg unit ( $S_{20,w}$ ).

Top: DNS-ribosome

Bottom: control

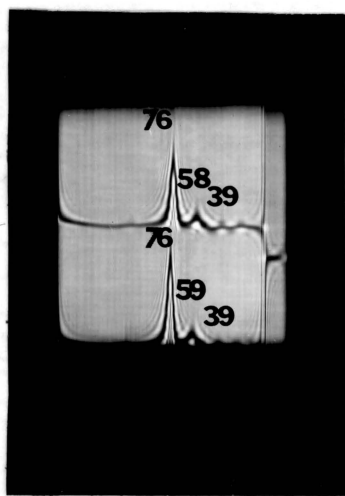


Figure 7. Analytical ultracentrifuge pattern of ribosomes (6mg/ml) dansylated at  $6 \times 10^{-4} \text{M}$   $\text{MgCl}_2$  concentration level and dialyzed vs. a buffer containing  $5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$ , 0.025M Tris-HCl (pH 7.8), 0.020M KCl, and 0.006M 2-mercaptoethanol. Schlieren patterns of control and dansylated ribosomes were taken at a phase angle of  $50^\circ$  and 15 minutes after the centrifuge reached 39,460 rpm at  $3.6^\circ$ . Sedimentation was from right to left. Numbers in figure are sedimentation coefficients in svedberg units ( $S_{20,w}$ ).

Top: DNS-ribosome

Bottom: control

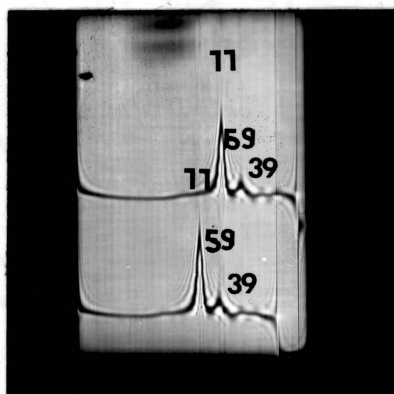
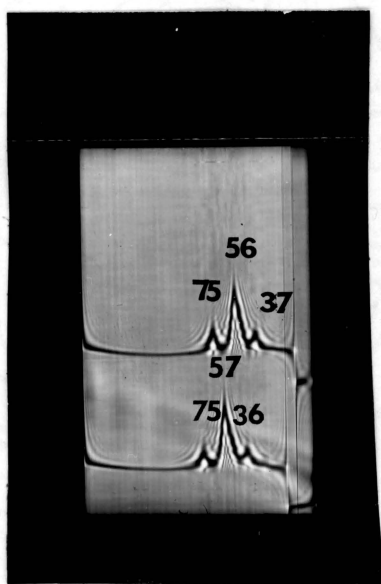


Figure 8. Analytical ultracentrifuge pattern of ribosomes (6mg/ml) dansylated at  $6 \times 10^{-4} \text{M}$   $\text{MgCl}_2$  concentration level and dialyzed vs. a buffer containing  $6 \times 10^{-4} \text{M}$   $\text{MgCl}_2$ , 0.025M Tris-HCl (pH 7.8), 0.020M KCl, and 0.006M 2-mercaptoethanol. Schlieren patterns of control and dansylated ribosomes were taken at a phase angle of  $50^\circ$  and 12 minutes after the centrifuge reached 39,460 rpm at  $6.7^\circ$ . Sedimentation was from right to left. Numbers in figure are sedimentation coefficients in svedberg unit ( $S_{20,w}$ ).

Top: DNS-ribosome

Bottom: control



(Figure 7) or  $5 \times 10^{-3} \text{M}$  (Figure 4) had same sedimentation pattern, when they were dialyzed at  $6 \times 10^{-4} \text{M}$   $\text{MgCl}_2$  concentration. Thus, the  $\text{MgCl}_2$  concentration of dansylation may not affect the sedimentation patterns of the dansylated ribosomes. Effect of magnesium concentration on the degree of dansylation was not investigated in the present work.

In order to check whether or not celite had any effect on ribosome sedimentation, dissociation or reassociation, and also to examine the  $\text{MgCl}_2$  concentration effect on the pine seed ribosome systems, sedimentation patterns of pine seed ribosomes at  $\text{MgCl}_2$  concentrations of  $5 \times 10^{-3} \text{M}$ ,  $1.5 \times 10^{-3} \text{M}$ ,  $6 \times 10^{-4} \text{M}$ , and  $1 \times 10^{-4} \text{M}$  EDTA (ethylenediaminetetraacetate) were tested (other components of each buffer medium were same as the standard buffer B) with celite omitted. Comparisons of the sedimentation patterns in Figure 9 and 10 with those corresponding sedimentation patterns in Figure 3, 6, and 8 leads to the conclusion that the celite treatment of ribosomes does not affect their sedimentation patterns. Figure 10 shows the break down of ribosomal subunits into smaller particles, when  $\text{Mg}^{++}$  is depleted.

#### The effect of ribonuclease on ribosomes and ribosomal subunits

As shown in Figure 11, 12, and 13, 80S ribosomes, and 60S and 40S subunits are all sensitive to ribonuclease at  $\text{MgCl}_2$  concentrations less than  $5 \times 10^{-3} \text{M}$ . However, the degree of sensitivity to ribonuclease varies according to the ribosome species, 80S ribosome being the most resistant and the 40S subunit the least resistant under all  $\text{Mg}^{++}$  levels tested, as shown by the chromicity change at 260 nm. The difference in ribonuclease sensitivity among the 80S ribosome, 60S, and 40S subunits becomes less as the  $\text{Mg}^{++}$  concentration level is lowered, while the ribonuclease



Figure 9. Analytical ultracentrifuge patterns of ribosomes (6mg/ml) dialyzed vs. a buffer containing 0.025M Tris-HCl (pH 7.8), 0.020M KCl, 0.006M 2-mercaptoethanol, and  $\text{MgCl}_2$  as indicated below. Schlieren patterns were taken at a phase angle of  $50^\circ$  and 18 minutes after the centrifuge reached 39,460 rpm at  $4.0^\circ$ . Sedimentation was from right to left. Numbers in figure are sedimentation coefficients in svedberg unit ( $S_{20,w}$ ).

Top: at  $5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$

Bottom: at  $1.5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$

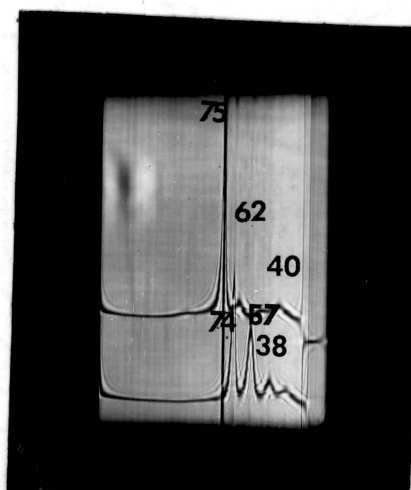


Figure 10. Analytical ultracentrifuge patterns of ribosomes (6mg/ml) dialyzed vs. a buffer containing 0.025M Tris-HCl (pH 7.8), 0.020M KCl, 0.006M 2-mercaptoethanol, and MgCl<sub>2</sub> concentration or EDTA concentration indicated below. Schlieren patterns were taken at a phase angle of 50° and 14 minutes after the centrifuge reached 39,460 rpm at 7.9°. Sedimentation was from right to left. Numbers in figure are sedimentation coefficients in svedberg unit ( $s_{20,w}$ ).

Top: at  $6 \times 10^{-4}$  M MgCl<sub>2</sub>

Bottom: at  $1 \times 10^{-4}$  M EDTA

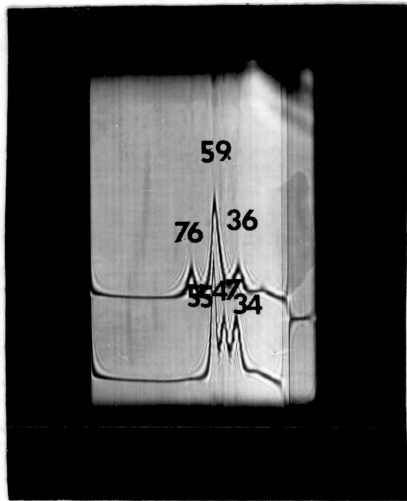


Table II Time (in minutes) required for completion of digestion, and hyperchromicity (in percent) at the plateau of ribonuclease digestion in a buffer containing 0.025M Tris-HCl (pH 7.8), 0.020M KCl, 0.006M 2-mercaptoethanol, and magnesium concentration at the desired levels as indicated in the Table.

ribosome species	Mg concentration level					
	$5 \times 10^{-3} \text{M}$		$1 \times 10^{-4} \text{M}$		$1 \times 10^{-4} \text{M}$ EDTA	
	hyper-chromicity	Time	hyper-chromicity	Time	hyper-chromicity	Time
80S	48	13	67	10	87	4
60S	43	9	69	7	65	4
40S	53	5	65	4	54	3
80S DNS-ribosomes	50	7	225	7	190	4

Figure 11. Effect of ribonuclease on ribosomes and ribosomal subunits at  $5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$ . A portion of 0.03 ml ribosome suspension in buffer B was mixed with 3 ml of  $5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$ , 0.025M Tris-HCl (pH 7.8), 0.02M KCl, and 0.006M 2-mercaptoethanol. The absorbance was followed at 260 nm. Detailed experimental conditions are described in Materials and Methods. Indicated ribosome species are those added into the reaction medium.

—●—●— = 80S ribosomes

—○—○— = 60S ribosomes

····○····○···· = 40S ribosomes

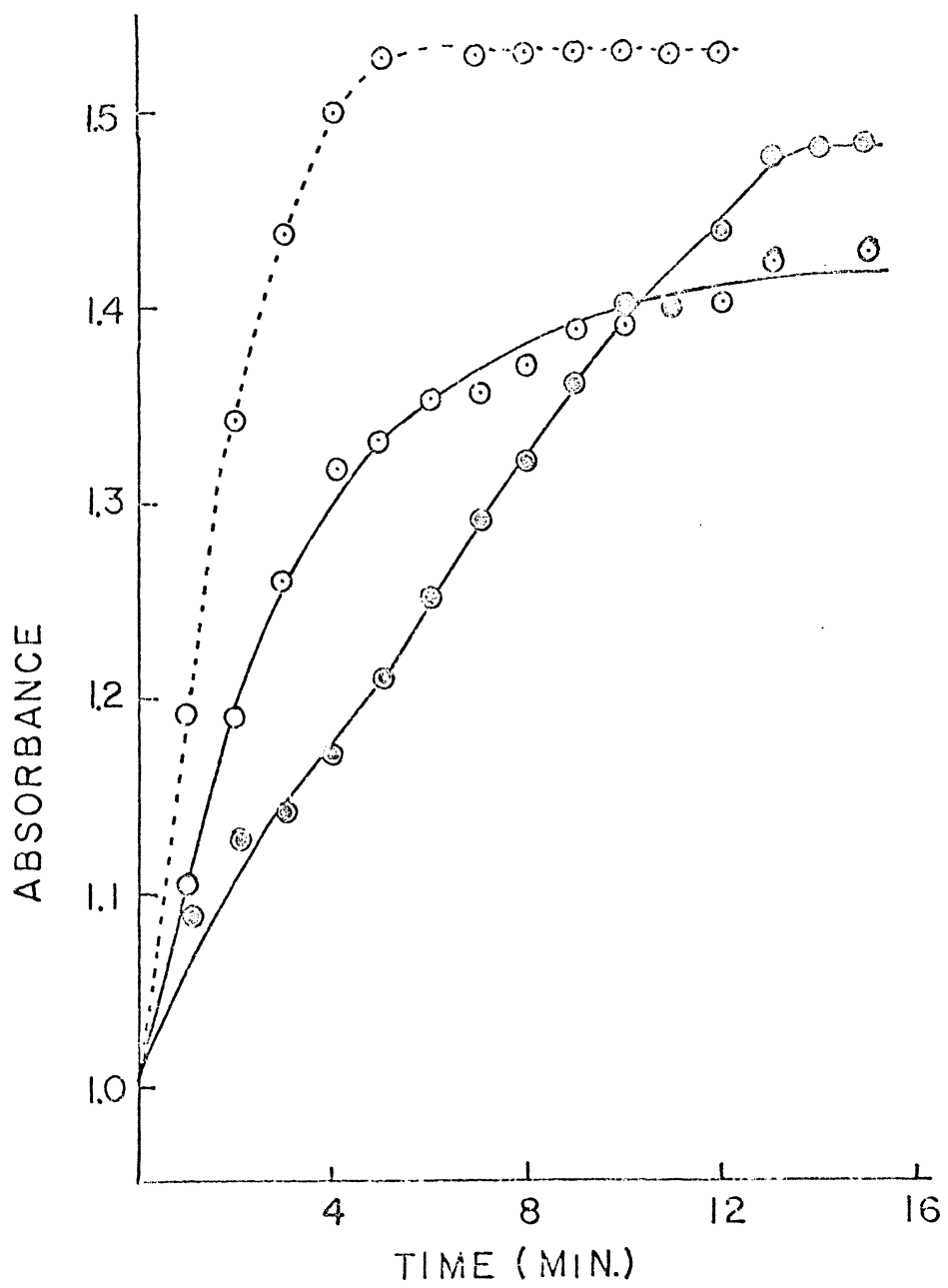


Figure 12. Effect of ribonuclease on ribosomes and ribosomal subunits at  $1 \times 10^{-4} \text{M}$   $\text{MgCl}_2$ . A portion of 0.03ml ribosome suspension in buffer B was mixed with 3 ml of  $5 \times 10^{-5} \text{M}$   $\text{MgCl}_2$ , 0.025M Tris-HCl (pH 7.8), 0.02M KCl, 0.006M 2-mercaptoethanol. The absorbance was followed at 260 nm. Detailed experimental conditions are described in Materials and Methods. Indicated ribosome species are those added into the reaction medium.

—●—●— = 80S ribosomes

—○—○— = 60S ribosomes

---○---○--- = 40S ribosomes



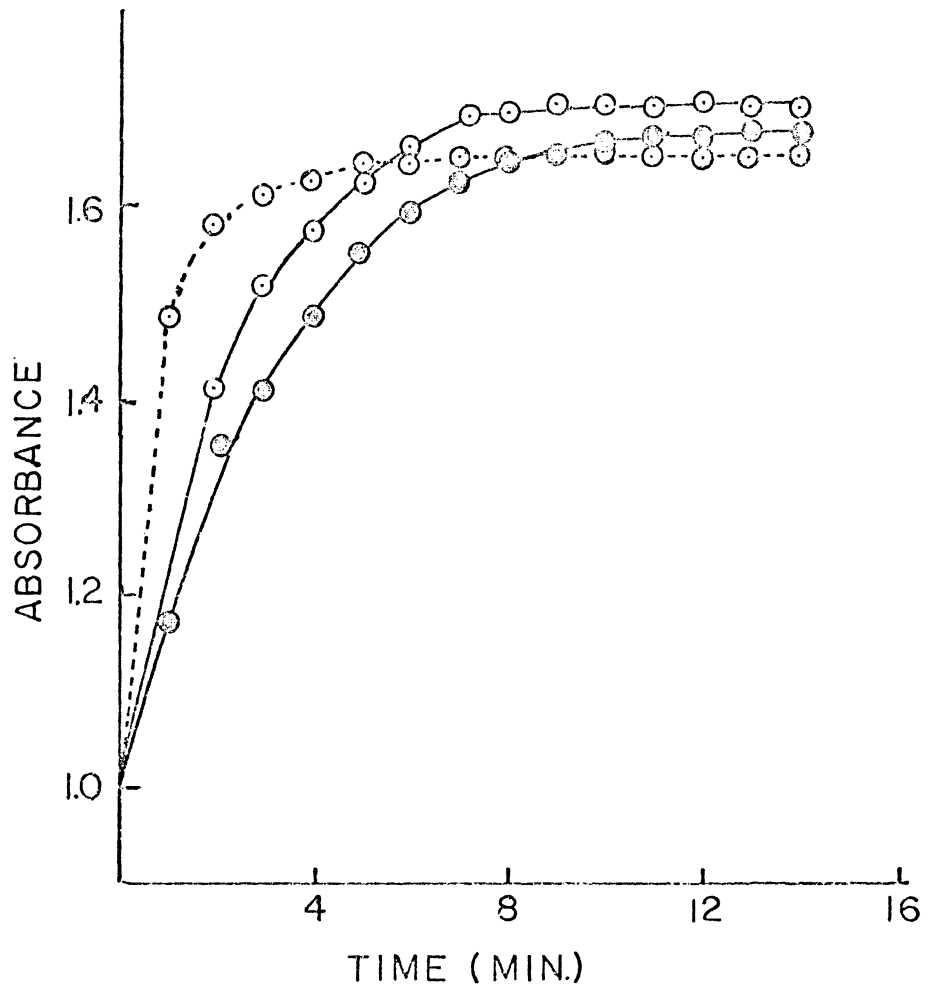
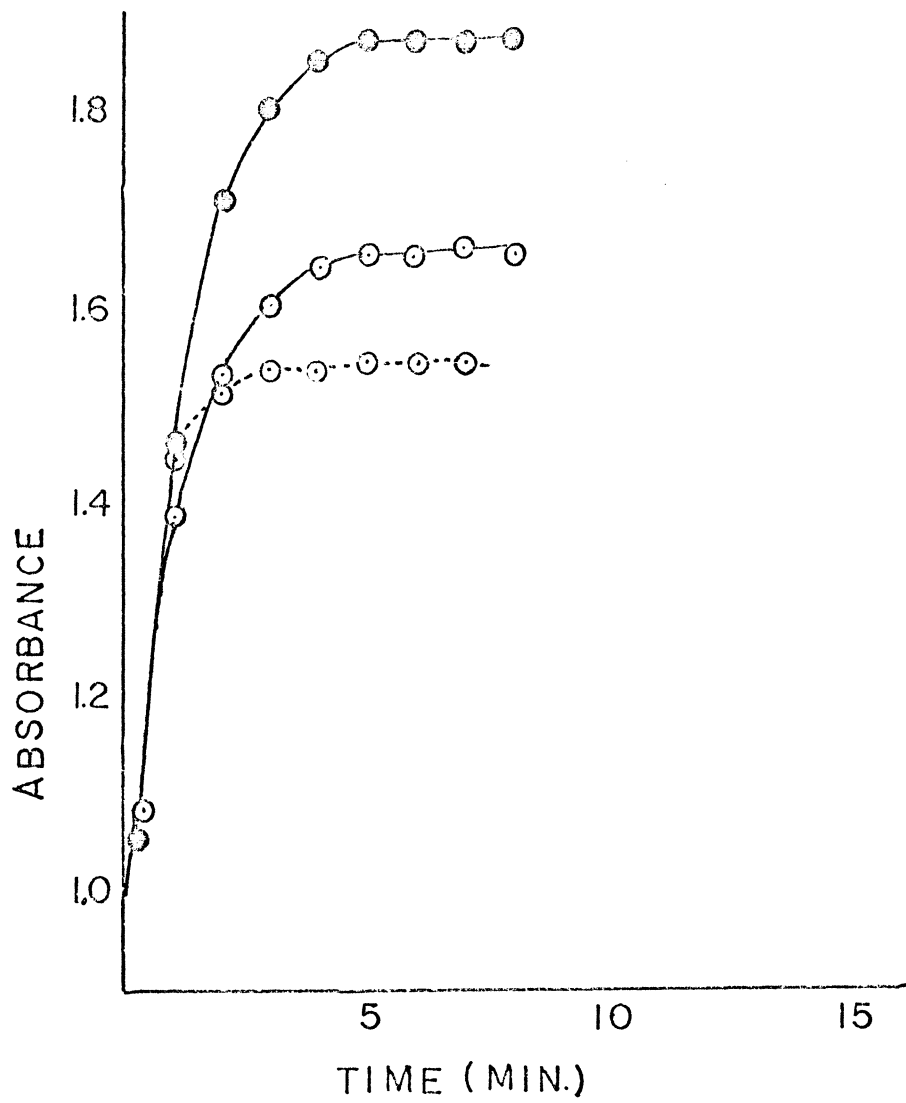


Figure 13. Effect of ribonuclease on ribosomes and ribosomal subunits at the depletion of magnesium with  $1 \times 10^{-4} \text{M}$  EDTA. A portion of 0.03 ml ribosome suspension in buffer B was mixed with 3 ml of  $1 \times 10^{-4} \text{M}$  EDTA, 0.025M Tris-HCl (pH 7.8), 0.02M KCl, and 0.006M 2-mercaptoethanol. The absorbance was followed at 260 nm. Detailed experimental conditions are described in Materials and Methods. Indicated ribosome species are those added into the reaction medium.

—●—●— = 80S ribosomes

—○—○— = 60S ribosomes

--○--○-- = 40S ribosomes



sensitivity of all ribosomal species (80S, 60S, and 40S) are increased as the  $Mg^{++}$  concentration decreases. The time required to reach the plateau and the hyperchromicity at the plateau for the 80S ribosomes, 60S subunit, 40S subunit under different  $Mg^{++}$  concentration levels are listed in Table II. The average hyperchromicity for all ribosomal species under all  $Mg^{++}$  concentration levels tested was about 61%.

#### Ribonuclease sensitivity of DNS-ribosomes

As shown in Figure 14, the hyperchromicity for DNS-ribosomes takes place faster than that for normal ribosomes (cf Figure 11, 12, and 13) at all  $Mg^{++}$  concentration levels, and the times required to reach plateau for DNS-ribosomes are shorter than for normal ribosomes for different  $Mg^{++}$  concentration levels (compare Figure 14 with 80S ribosome hyperchromicity curves in Figure 11, 12, and 13). The time required for DNS-ribosome to reach plateau was 7 minutes, 7 minutes and 4 minutes at  $5 \times 10^{-3}M$   $MgCl_2$ ,  $1 \times 10^{-4}M$   $MgCl_2$  and  $1 \times 10^{-4}M$  EDTA respectively, whereas 13 minutes, 10 minutes, and 4 minutes respectively for normal ribosomes at the same  $Mg^{++}$  concentrations (Table II).

#### Temperature-absorbance profiles of 80S ribosomes, 40S subunit, and 80S DNS-ribosomes at 260 nm

Figure 15 shows the absorbance at 260 nm, as a function of temperature, of 80S ribosomes, 40S subunit ribosomes and 80S DNS-ribosomes in  $1.5 \times 10^{-3}M$   $MgCl_2$ , 0.025M Tris-HCl (pH 7.8), 0.020M KCl and 0.006M 2-mercaptoethanol. In this figure, one can see that the  $T_m$  values of the DNS-ribosome and normal ribosome are  $39.0^\circ$  and  $41.1^\circ$ , respectively; the  $T_m$  value of the ribosome was lowered  $2^\circ$  by dansylation. The 40S subunit ribosome had no such discernable  $T_m$  over the temperature range tested.

Figure 14. Effect of ribonuclease on 80S DNS-ribosomes at different magnesium concentration levels ( $5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$ ,  $1 \times 10^{-4} \text{M}$   $\text{MgCl}_2$ , and  $1 \times 10^{-4} \text{M}$  EDTA). A portion of 0.03 ml DNS-ribosome suspension in buffer B was mixed with 3 ml of 0.025M Tris-HCl (pH 7.8), 0.020M KCl, 0.006M 2-mercaptoethanol, and  $\text{MgCl}_2$  or EDTA of the desired concentration as indicated below. The absorbance was followed at 260 nm.

—●—●— = at  $5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$

—○—○— = at  $5 \times 10^{-5} \text{M}$   $\text{MgCl}_2$

—○—○— = at  $1 \times 10^{-4} \text{M}$  EDTA

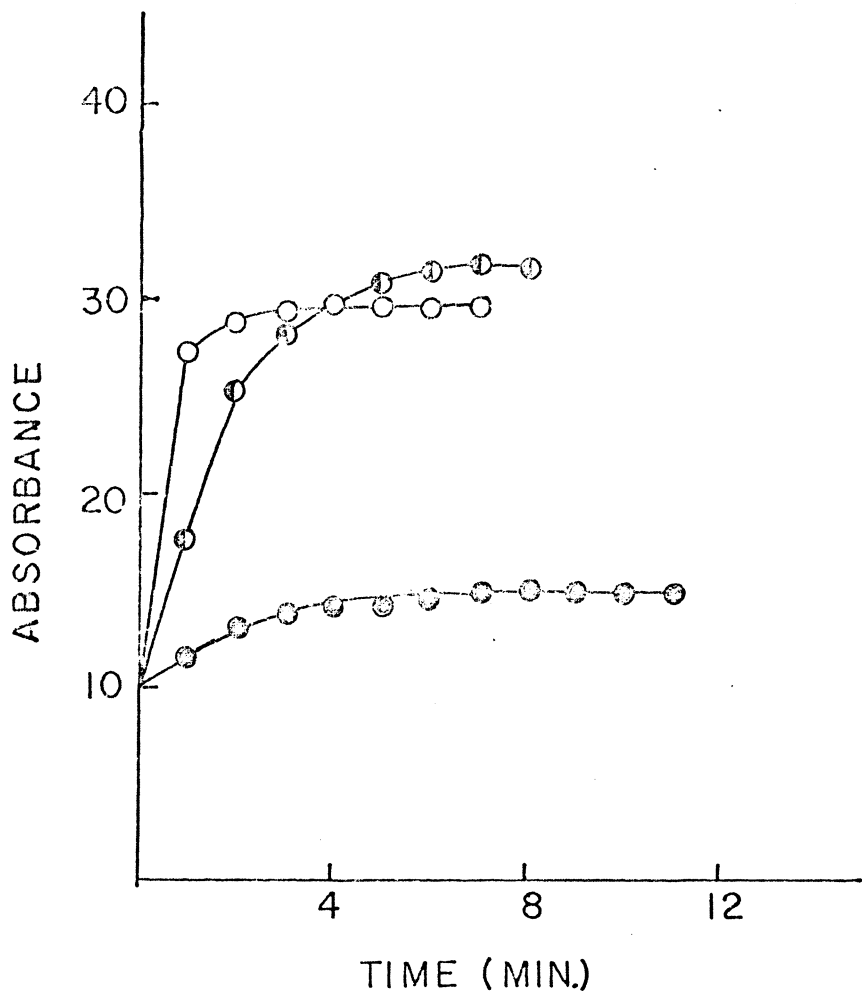
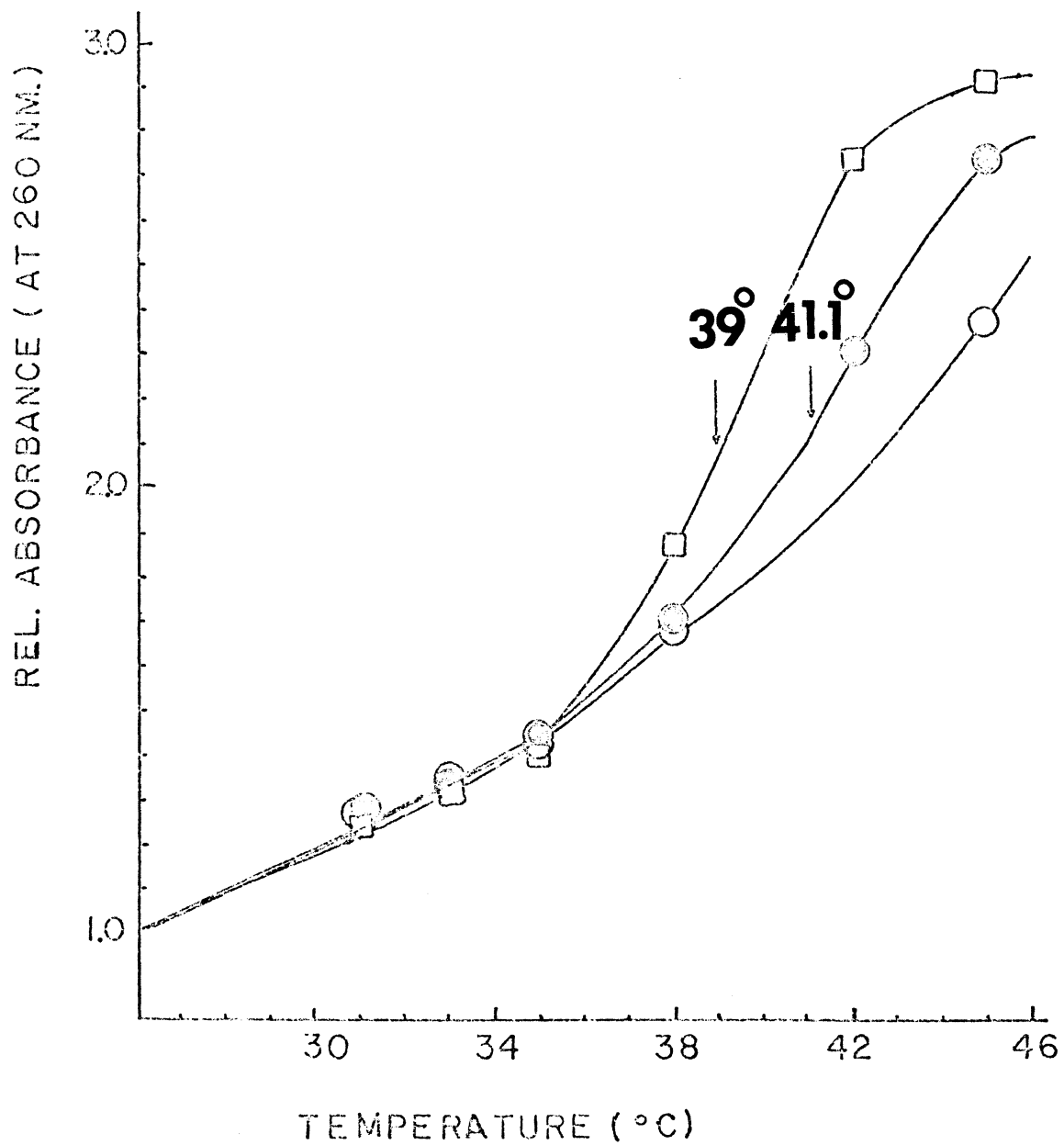


Figure 15. Temperature-absorbance profiles of 80S ribosomes, 80S DNS-ribosomes, and 40S subunit ribosomes in  $1.5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$ , 0.025M Tris-HCl (pH 7.8), 0.020M KCl, and 0.006M 2-mercaptoethanol. The absorbance was followed at 260 nm. The initial starting absorbances, 0.5-0.7 were normalized to 1.0.

—●—●— = 80S ribosomes

—■—■— = DNS-ribosomes

—○—○— = 40S ribosomes





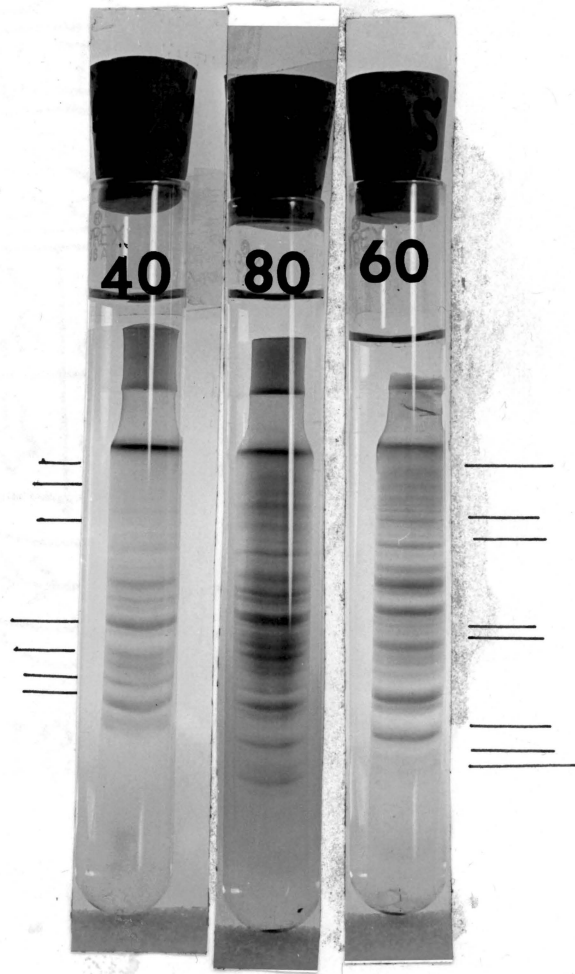
Above  $51^{\circ}$ , the ribosomes aggregated under these experimental conditions, and it was impossible to get thermal hyperchromicity higher than this temperature. Such aggregation phenomena in temperature-absorbance profile study in the presence of  $Mg^{++}$  have been reported with other systems (Zubay and Wilkins, 1960; Ohtaka and Uchida, 1963; Cotter et al., 1967). The slope of differentiated melting curve at the  $T_m$  was 0.22 for DNS-ribosome, and 0.165 for normal ribosome. Thus, the slope of differentiated melting curve of the ribosome was increased by 0.057 by dansylation.

#### Disc gel electrophoresis

Figure 16 shows disc gel electrophoresis columns for r-proteins from 80S, 60S and 40S subunits. Line bars show on those protein bands, which are unique to one subunit. Some of the protein bands of each subunit do not overlap. The number of r-protein bands of the 80S, 60S and 40S particles are approximately 25, 19, and 15, respectively. See Table I for comparison with other systems. The difference between the number of bands of 80S ribosomes and the number of bands of 60S plus 40S may be due to overlapping bands. Figure 17 shows the electrophoresis patterns of r-proteins obtained by LiCl-urea method, acetic acid method, and those from  $NH_4$ -ribosomes obtained by LiCl-urea method. Their patterns are similar. Thus, different methods of r-protein preparation produce same patterns on disc electrophoresis, and washing  $NH_4$ -ribosome with buffer B has no effect on patterns and numbers of bands, although proteins are removed in the washing procedure.

Figure 16. Polyacrylamide gel (15%) electrophoresis patterns at pH 4.5 of r-proteins from 80S ribosomes, 60S subunit, and 40S subunit. Migration was downwards, towards the cathode. Line bars indicate unique and nonoverlapping bands for each subunit species (60S and 40S).

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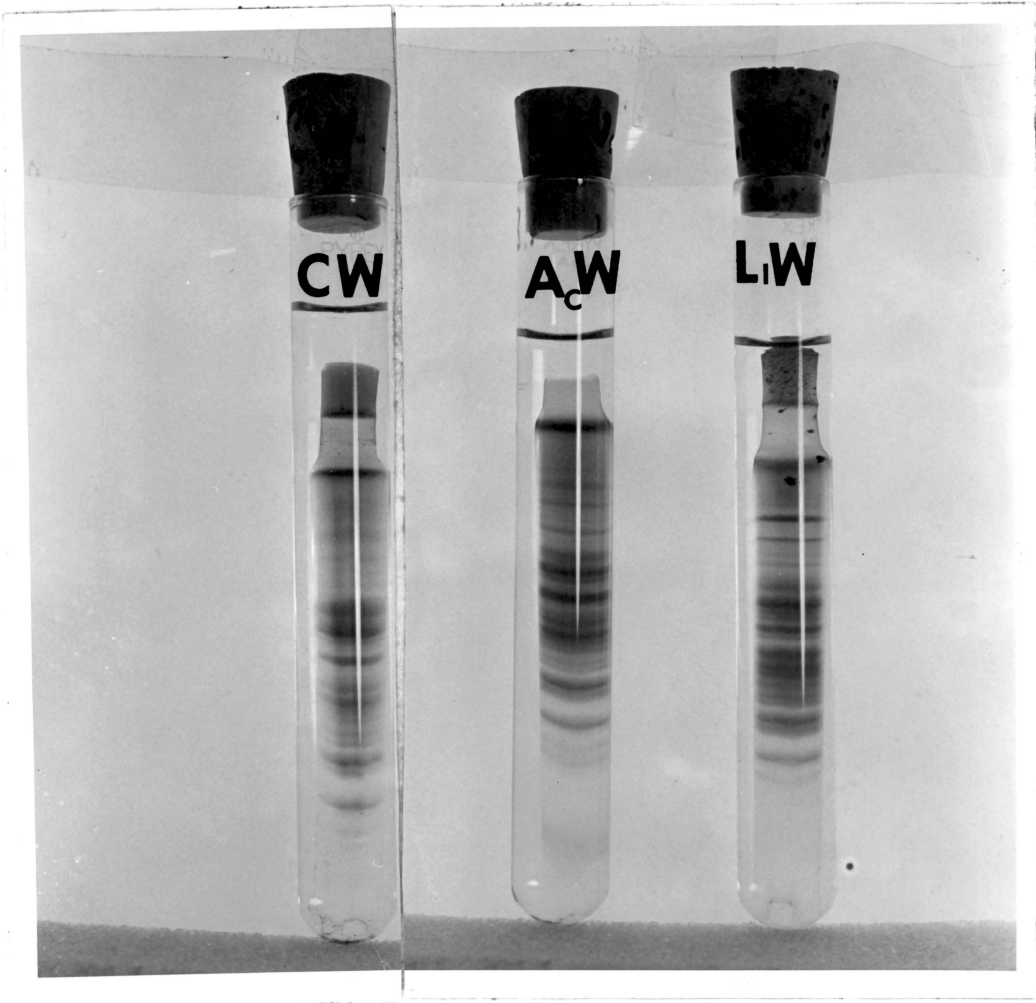
Figure 17. Polyacrylamide electrophoresis patterns at pH 4.5 of pine seed r-proteins prepared by the LiCl-urea, and the acetic acid methods, and the patterns of r-proteins extracted by the LiCl-urea method from  $\text{NH}_4$ -ribosome. Migration was downwards, toward the cathode.

AcW = ribosomal proteins by acetic acid method

LiW = ribosomal proteins obtained by LiCl-urea method

CW = ribosomal proteins from  $\text{NH}_4$ -ribosomes by LiCl-urea method

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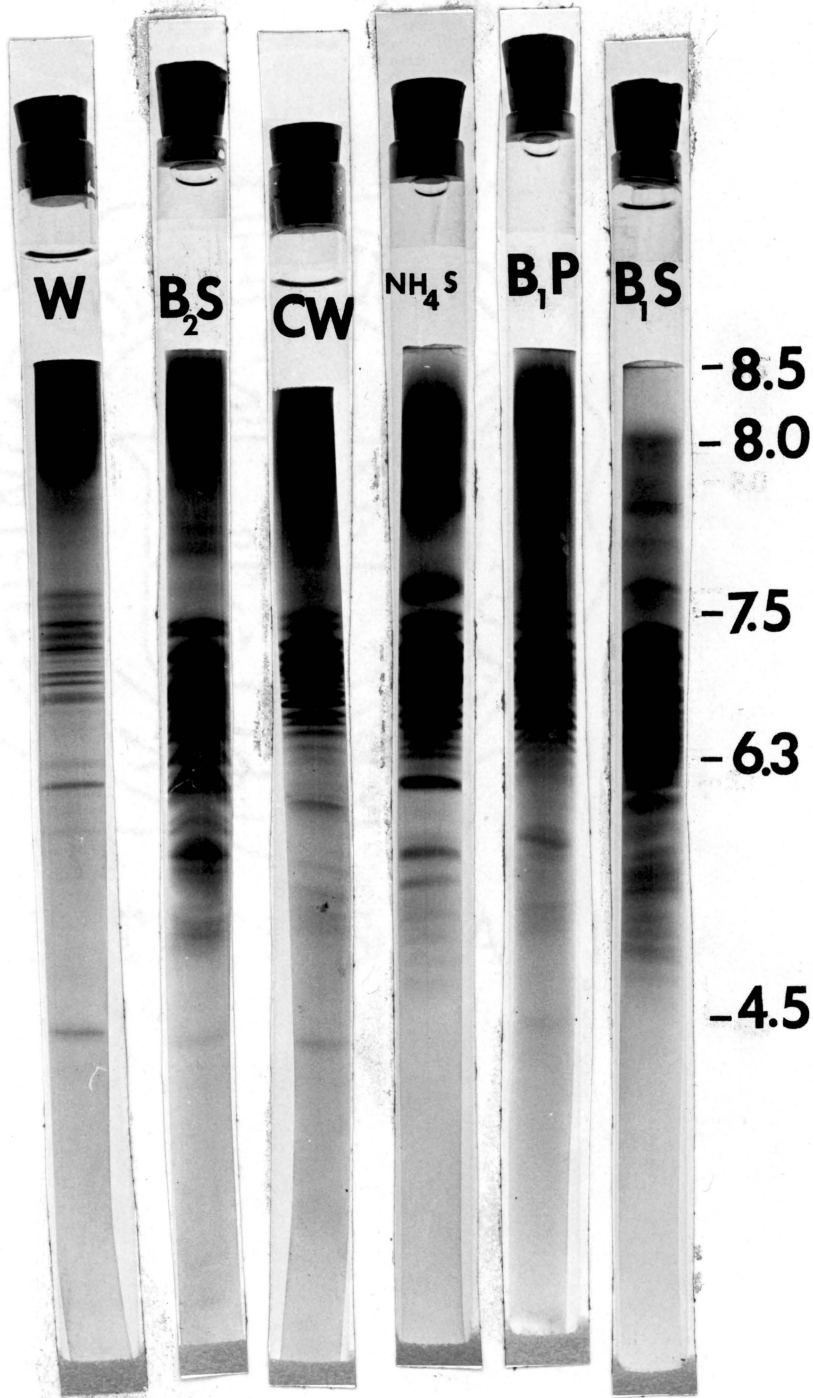


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### Isoelectric focusing

Isoelectric focusing patterns of proteins from each ribosomal pellet fraction and each wash supernatant fraction in the ribosome preparation process are shown in Figure 18. In the pictures, it is noteworthy that: majority r-proteins from ribosomal pellet fractions are concentrated between pH's of 8 and 8.5, and 6.3 and 7.5; degree of detachability of r-proteins from ribosomes differ depending upon their pI's (isoelectric point); the most basic (pI 8-8.5) and the most acidic (pI 4.5) r-protein components are the most strongly bound, as they are not detached in the initial wash supernatant fraction (B<sub>1</sub>S). These acidic and basic proteins become detached by subsequent washing processes while the neutral r-proteins, whose pI values lie between 6.3 and 7.5 can be easily detached and they appear in high concentration in the initial wash supernatant. However, these readily detachable neutral proteins seem to be ribosomal, since most of them from supernatant fractions overlap with those from ribosomal pellet fractions, even in the case of extensively washed ribosomal pellets. On the whole, most r-protein patterns from supernatant fractions and ribosomal pellet fractions overlap and protein fractions did not appear to be completely lost during the wash procedures. The ribosomal pellets have only few bands with the acidic pH values and most of these were very basic. There was, however, a rather high concentration of an acidic component whose pI was 4.5. A more detailed study, especially for those basic r-proteins of high pI values (7.5-8.5), by the isoelectric focusing electrophoresis technique should be performed by using ampholites of shorter range of pH gradients according to the pI values of the group of

Figure 18. Isoelectric focusing of proteins from ribosomal pellets and from supernatant fractions of ribosomal washes. Numbers indicate pH values at which the bands were found. Identification of the sources of proteins are as follows: B<sub>1</sub>S, B<sub>1</sub>-supernatant; B<sub>1</sub>P, B<sub>1</sub>-ribosome; NH<sub>4</sub>S, NH<sub>4</sub>-supernatant; CW, NH<sub>4</sub>-ribosome; B<sub>2</sub>S, B<sub>2</sub>-supernatant; W, B<sub>2</sub>-ribosome. Protein samples were prepared as described in amino acid analysis.



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r-protein components which is to be investigated. Figure 19 shows the pH gradient along the polyacrylamide gel. This was used to estimate the pI values of each focused protein band. In Figure 20 60S and 40S r-protein patterns are compared. The smaller subunit, 40S, has lost the most acidic r-protein component whose pI value was 4.5, while the larger subunit, 60 does have the component. There were no other obvious differences in the isoelectric focusing electrophoresis patterns of r-proteins from 1M LiCl split free protein fraction and particulate fraction (Figure 21).

Ultraviolet spectra of supernatant fractions and ribosomal pellet fractions from the ribosomal preparation processes.

Prior to ultraviolet spectrum analysis, each supernatant, i.e., B<sub>1</sub>-supernatant, NH<sub>4</sub>-supernatant, and B<sub>2</sub>-supernatant, was centrifuged at 105,000 x g for 3 hours to eliminate any contaminating pellet particles. The surface of each ribosomal pellet (B<sub>1</sub>-ribosome, NH<sub>4</sub>-ribosome, and B<sub>2</sub>-ribosome) was gently rinsed with a small amount of buffer B, and suspended in buffer B. Then, each sample was dialyzed against buffer B before the ultraviolet spectral analysis. The U. V. spectral analysis was performed at room temperature, using buffer B as the reference medium. The ultraviolet spectra of supernatant fractions had their maximum absorption peak at 276 nm, while ribosomal pellet fractions had maximum absorption at 260 nm (see Figure 22 for B<sub>2</sub>-ribosome). In Table III are listed the absorbance ratios at the wavelengths (nm) 260 to 235 and 260 to 280, and the RNA and protein content (based on those absorbance data, employing a nomogram distributed by California Corporation for Biochemical Research), of each ribosomal pellet fractions. From these data and

Figure 19. pH gradient along the polyacrylamide gel column, which was used in the estimation of pI values of protein bands focused.

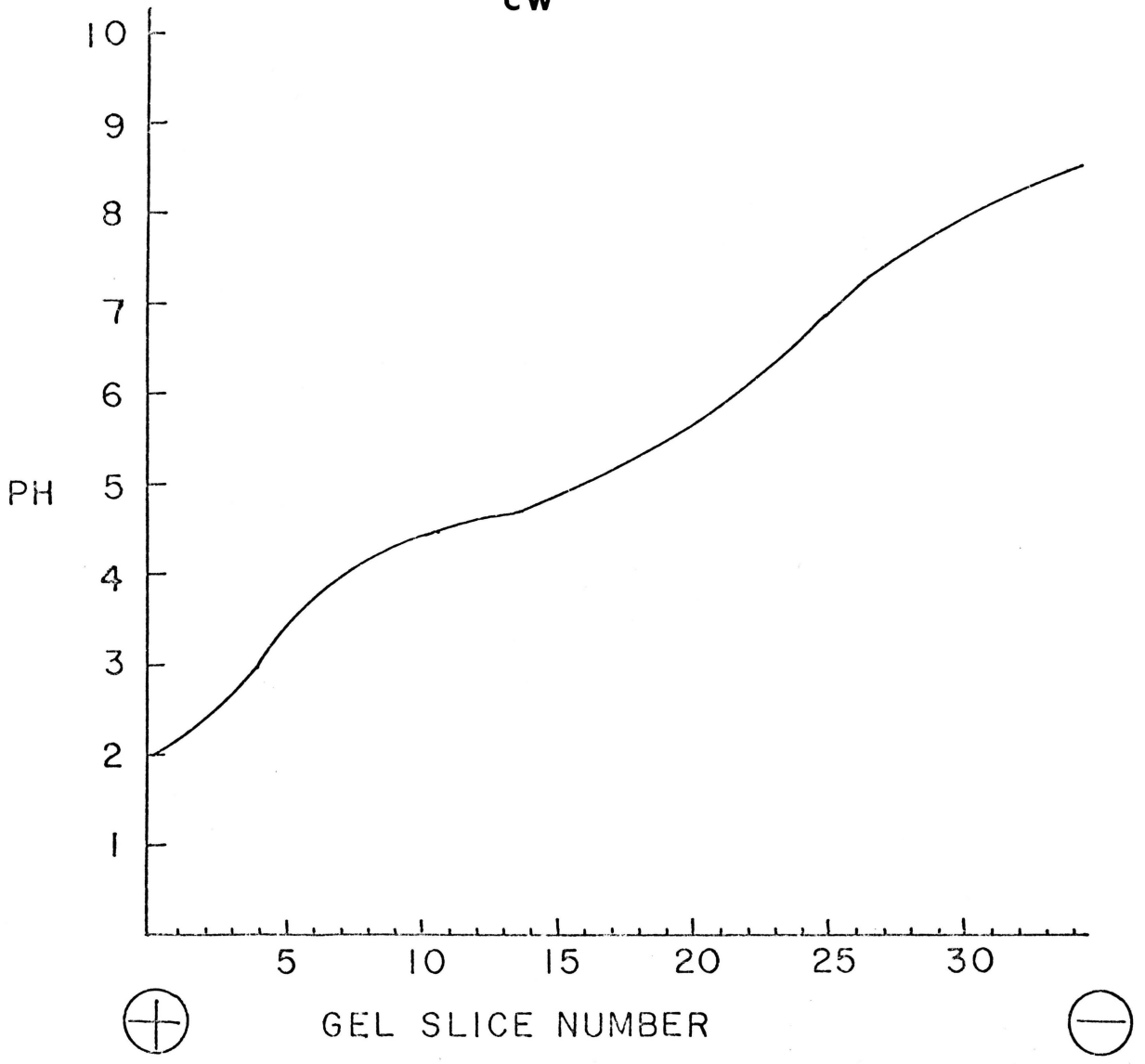
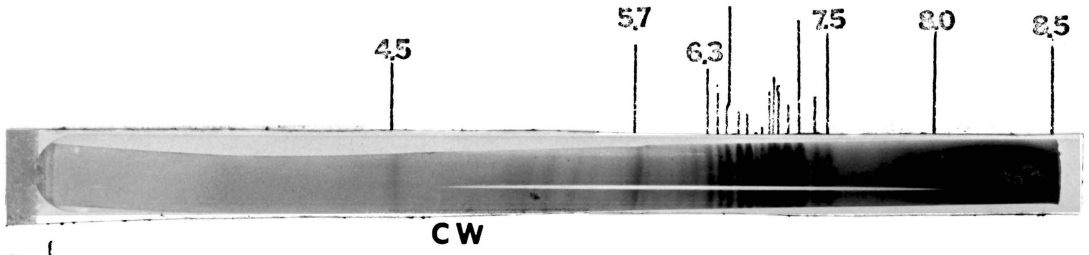
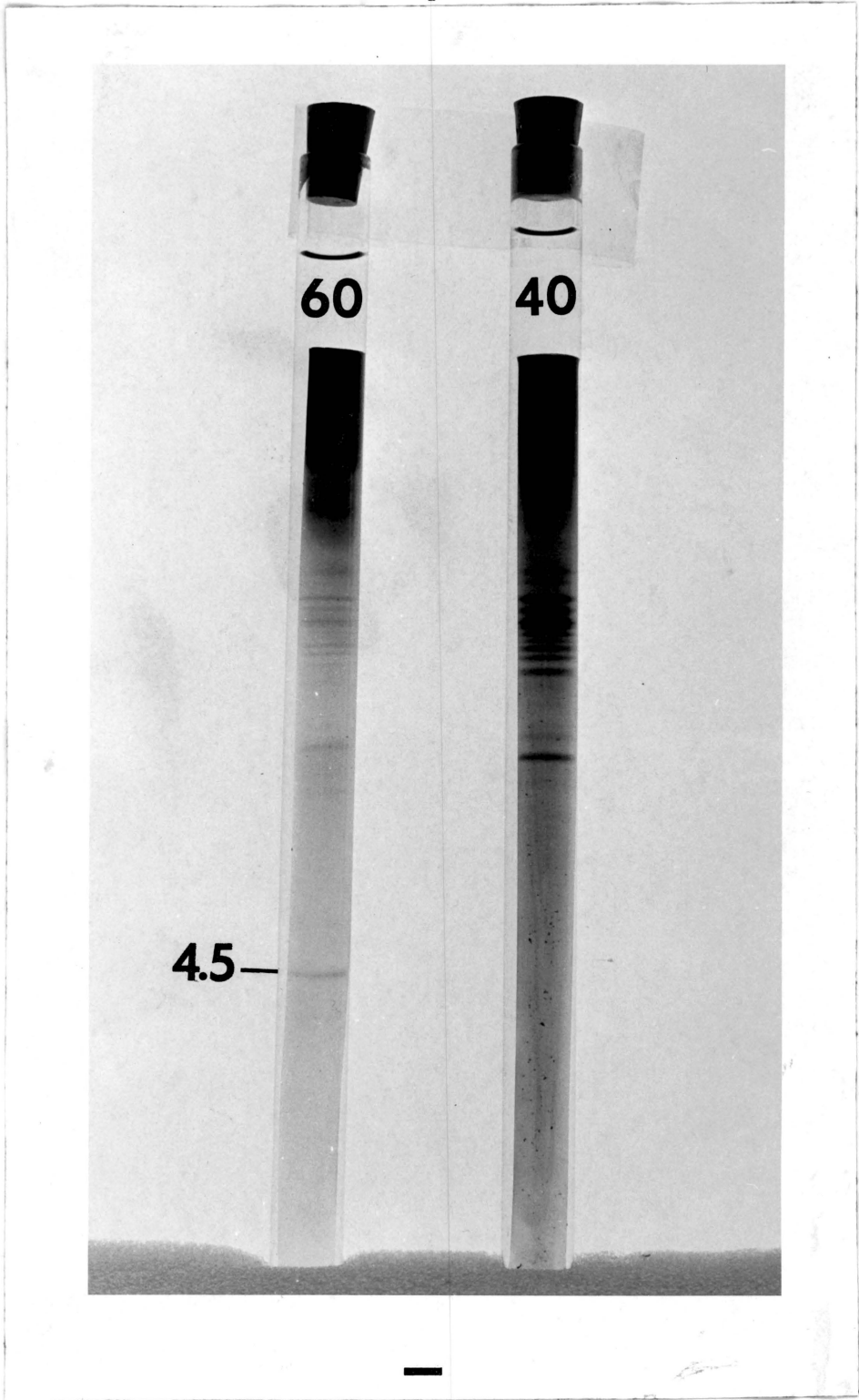


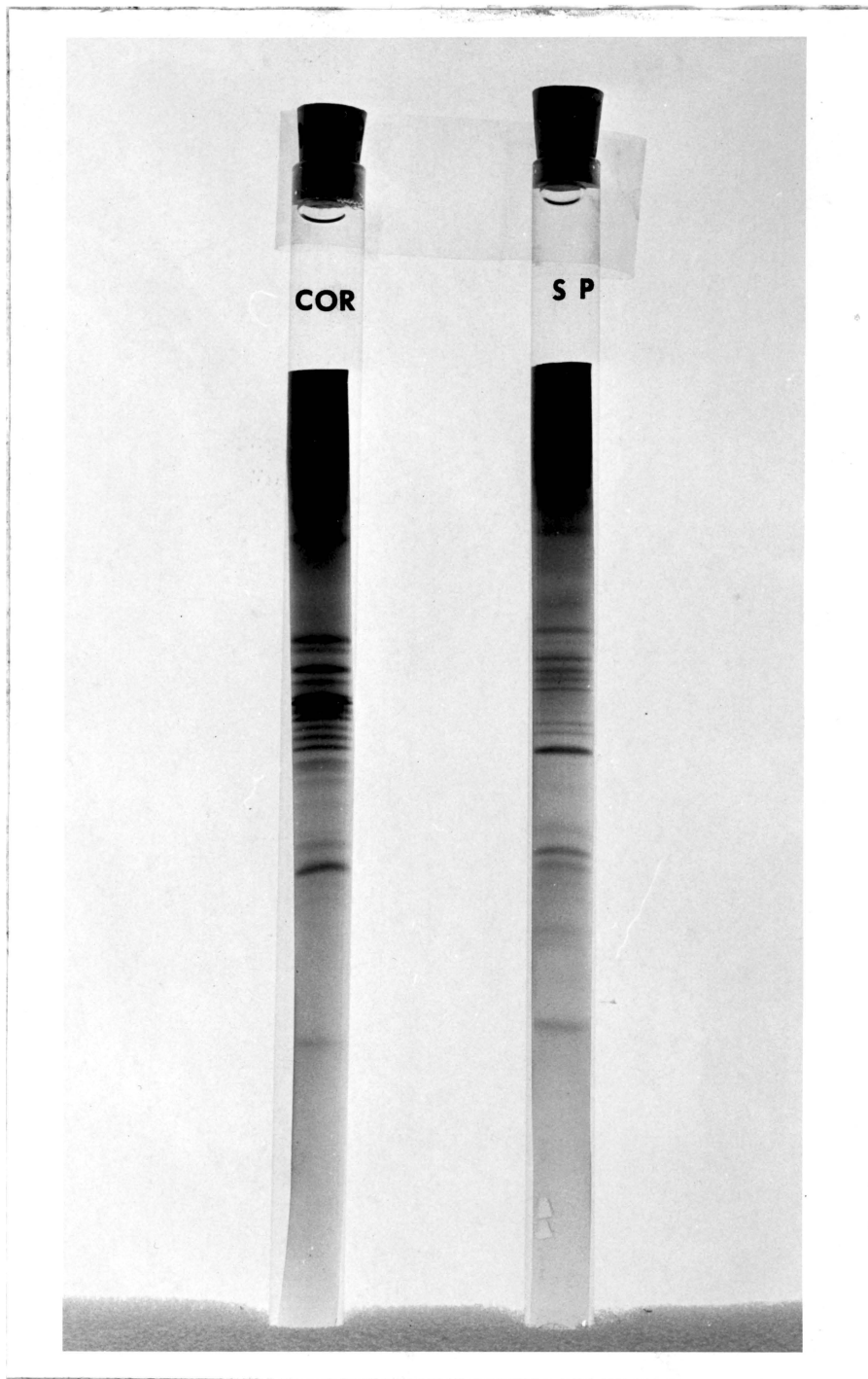
Figure 20. Comparison of the isoelectric focusing patterns of r-proteins from 40S and 60S particles, respectively.

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Figure 21. Isoelectric focusing of proteins from 1M LiCl-particulate fraction (cor) and 1M LiCl-split free protein (sp). Both samples were prepared as described in amino acid analysis.



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Figure 22. Ultraviolet spectrum of B<sub>2</sub>-ribosome



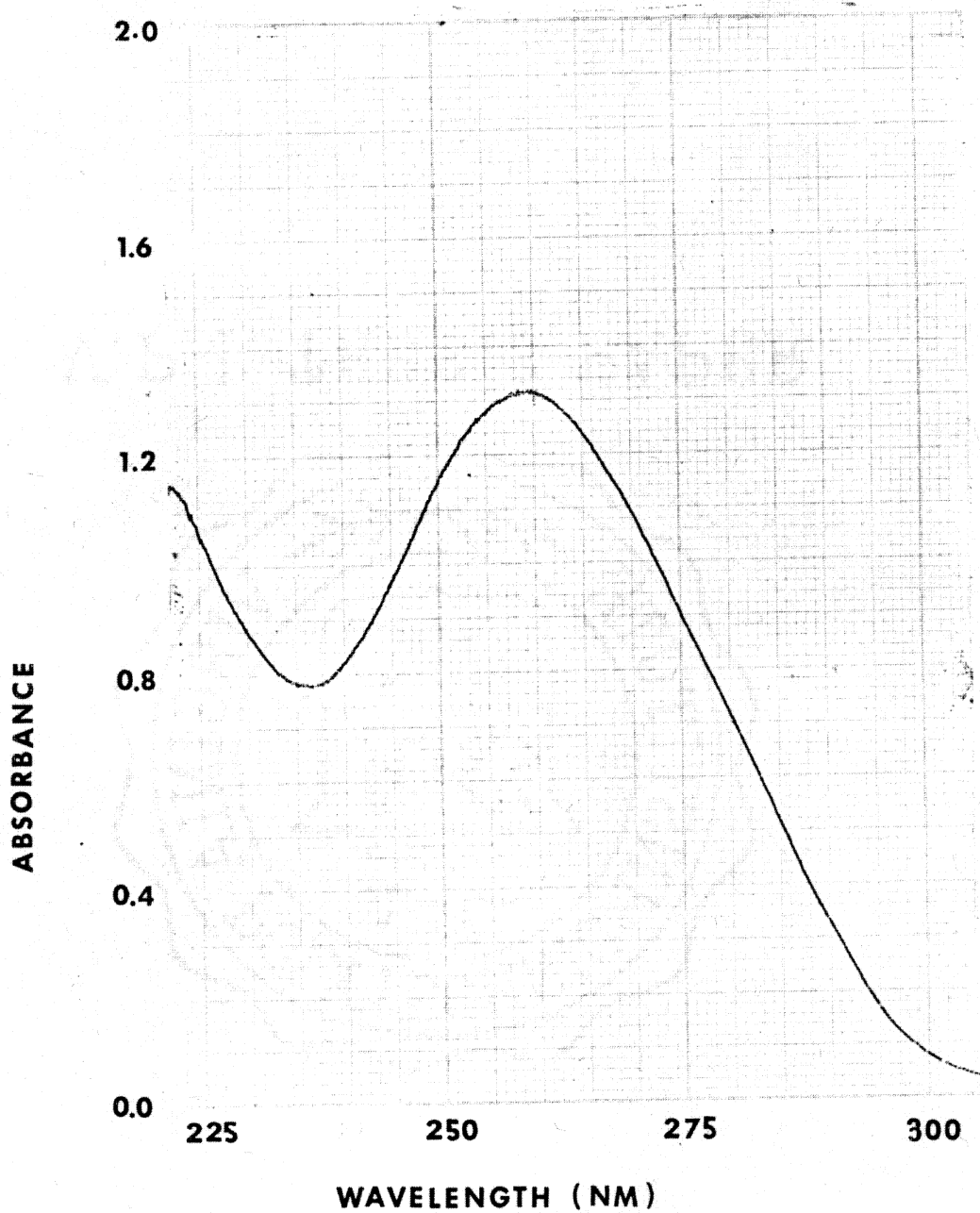


Table III Absorbance ratios: 260 nm to 235 nm, and 260 nm to 280 nm; and protein and RNA content of ribosomal pellet fractions. Protein and RNA content were estimated by the absorbances at 260 nm and 280 nm.

sample	260/235	260/280	% RNA	% protein
B <sub>1</sub> -ribosome	1.287	1.704	21	79
NH <sub>4</sub> -ribosome	1.497	1.756	25	75
B <sub>2</sub> -ribosome	1.719	1.840	33	67

spectra, one can see that, at each step of the washing procedure, chiefly proteins were eliminated. The ratio of the yields of proteins in the supernatants to the B<sub>2</sub>-supernatant was B<sub>1</sub>-sup.: NH<sub>4</sub>-sup.: B<sub>2</sub>-sup.: B<sub>2</sub>-ribosome of 240:85:1:427. These were estimated by weighing proteins obtained after dialysis, centrifugation and lyophilization as described in the method for the preparation of r-protein. Thus, the amount of detached protein was least in the B<sub>2</sub>-supernatant.

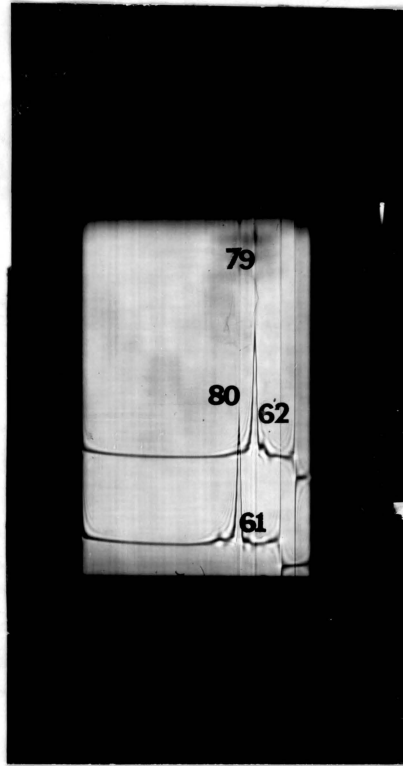
Structural integrity of ribosomal species, B<sub>1</sub>-ribosome, NH<sub>4</sub>-ribosome, and B<sub>2</sub>-ribosome

The detachment of considerable amount of proteins from ribosomal species during the washing processes was demonstrated in the previous section. In order to check whether or not the structural integrity had been maintained through the washing processes with detachment of proteins, the sedimentation patterns of the ribosomal species were compared. As shown in Figure 9, Top and Figure 23, the sedimentation patterns of B<sub>1</sub>-ribosome, NH<sub>4</sub>-ribosome, and B<sub>2</sub>-ribosome have no appreciable differences and all of them appear to have the integrity of normal ribosomes.

Dissociation of ribosome into subunits

The elution pattern of subunit ribosomes on 8-32 percent sucrose gradient made in  $6 \times 10^{-4} \text{M}$  MgCl<sub>2</sub>, 0.025M Tris-HCl (pH 7.8). 0.02M KCl is shown in Figure 24. The sedimentation pattern of the ribosome in this dissociation buffer is shown in Figure 10, Top. Figure 25 and 26 show ultraviolet spectra of 60S and 40S ribosomal subunits. The ratio of absorbances at 260 nm to 280 nm was 1.7 for both subunits. Estimation

Figure 23. Analytical ultracentrifuge patterns of B<sub>1</sub>-ribosomes (bottom picture) and NH<sub>4</sub>-ribosomes (top picture). Both of them (3mg/ml) were suspended in buffer B. Schlieren patterns were taken at a phase angle of 50° and at 12 minutes after the centrifuge reached 39,460 rpm at 4.0°. Sedimentation was from right to left. Numbers in figure are sedimentation coefficients in svedberg unit ( $s_{20,w}$ ).



79

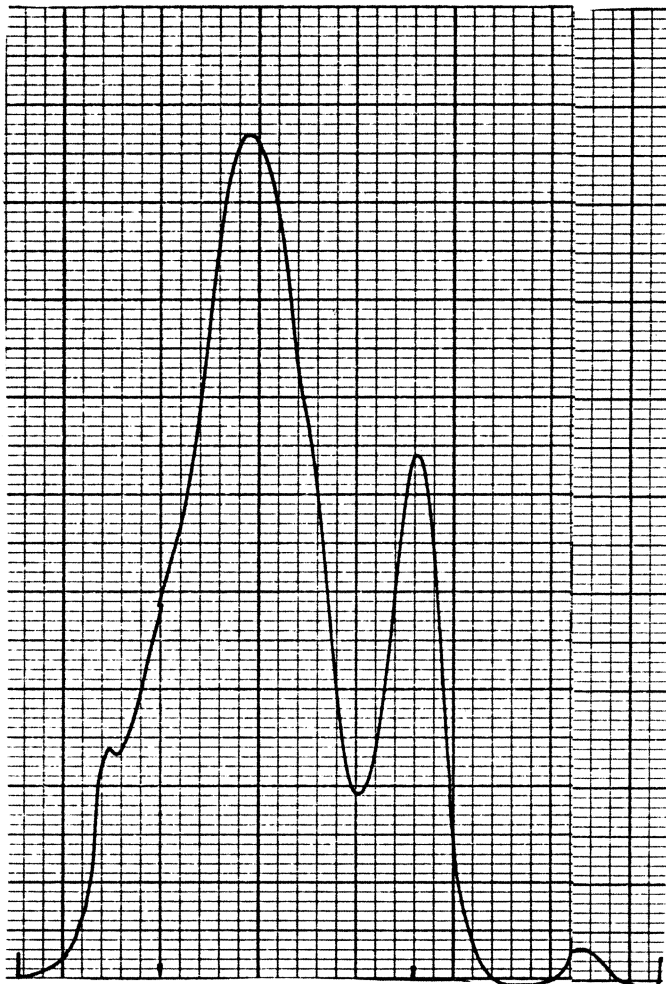
80

62

61

Figure 24. Elution pattern of 10 mg of ribosomal subunits on a 8-32 per cent sucrose gradient made in  $6 \times 10^{-4} \text{M}$   $\text{MgCl}_2$ , 0.025M Tris-HCl (pH 7.8), 0.020M KCl, after centrifugation for 9 hours at 23,000 rpm in the Type SW 27 rotor of the Beckman Model L-2 65B Ultracentrifuge at  $4^\circ$ .

↑ ABSORBANCE ( AT 254 NM ) INCREASE →



32

16

ML

BOTTOM

TOP

Figure 25. Ultraviolet spectrum of 60s subunit ribosome. the subunit ribosome was suspended in buffer B, and the spectrum was scanned at room temperature, using buffer B as a reference medium.



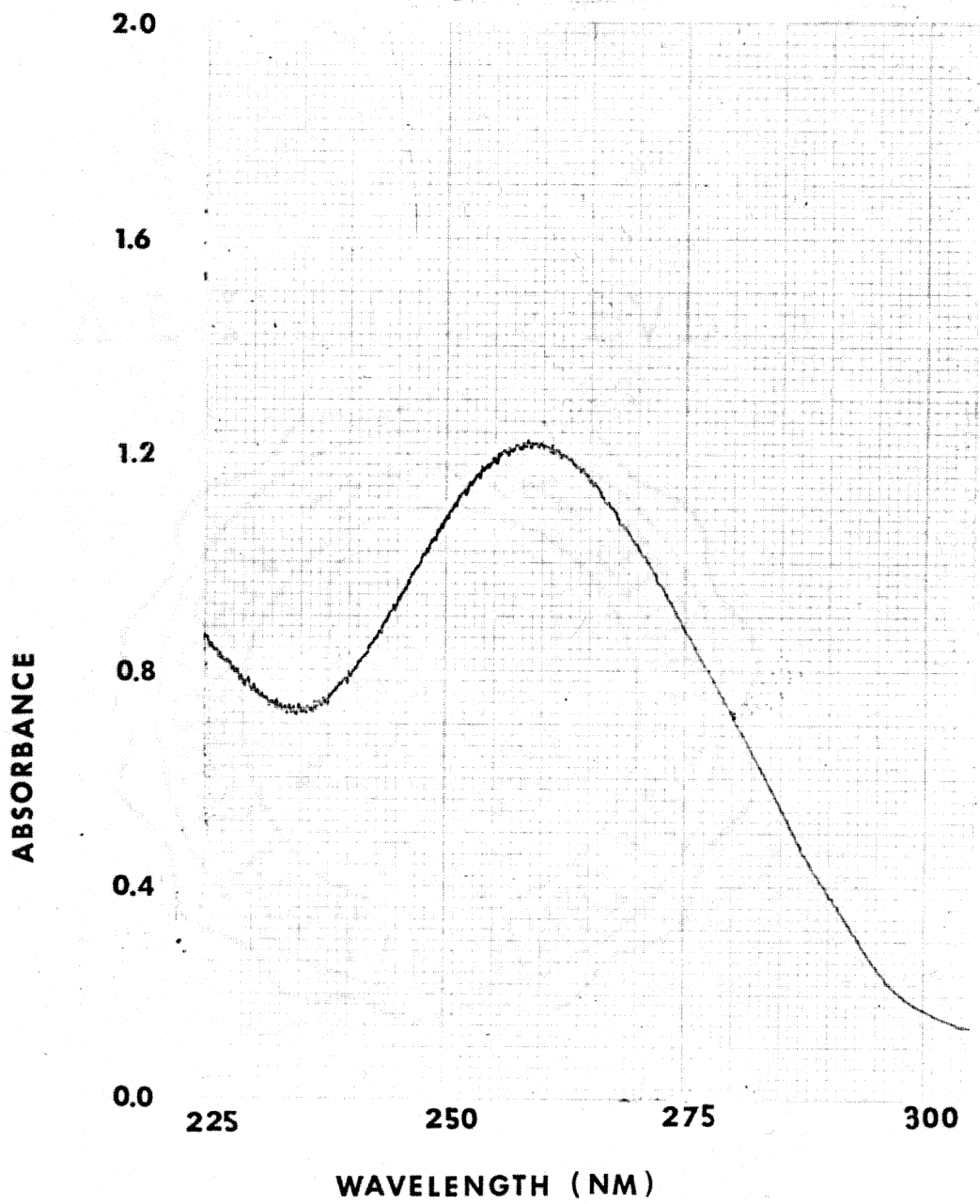
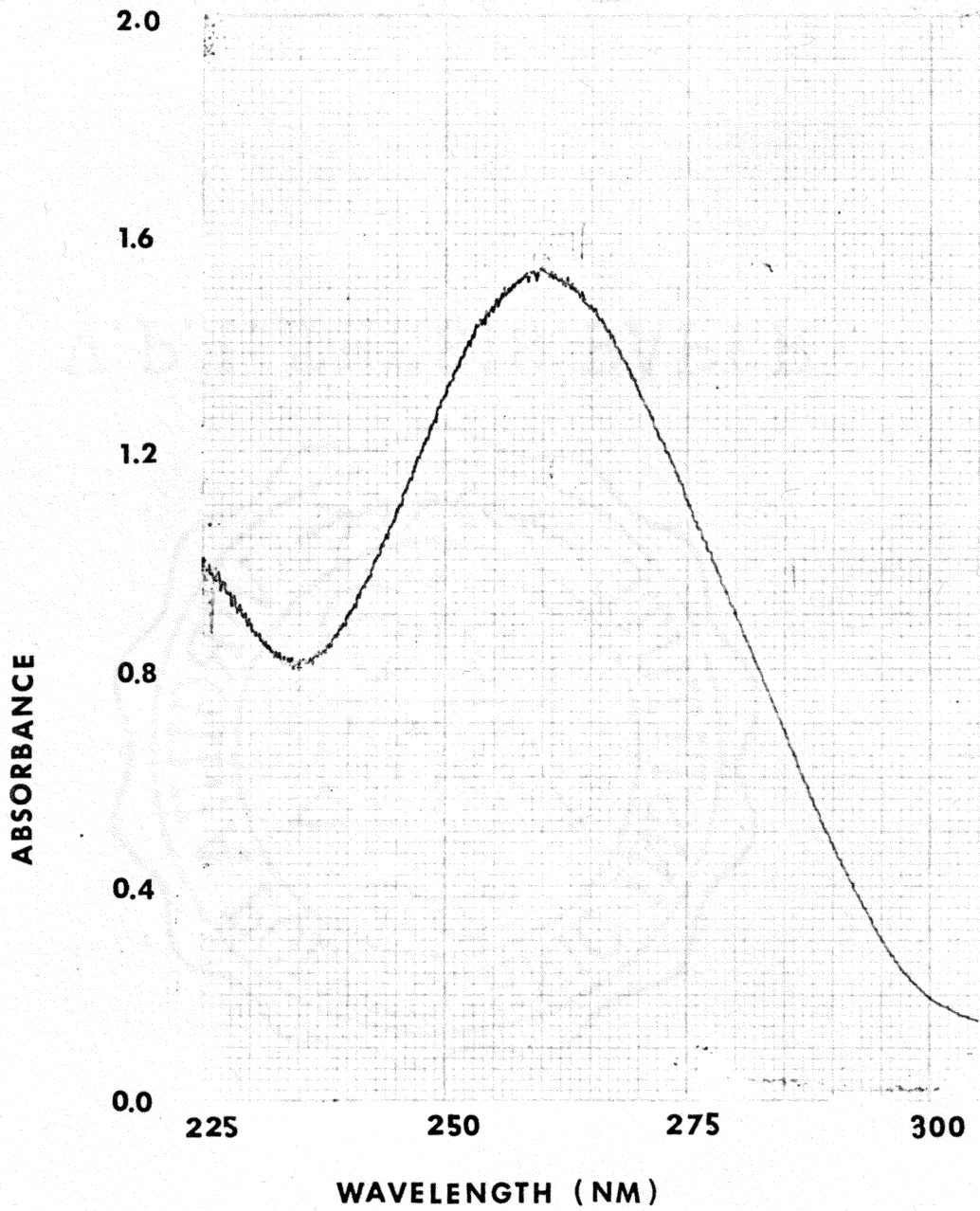


Figure 26. U.V. spectrum of the 40s particle. The subunit ribosome was suspended in buffer B, and the U.V. spectrum was scanned at room temperature using buffer B as reference medium.



of the composition of ribosomes were as follows:

<u>species of ribosomes</u>	<u>RNA (%)</u>	<u>protein (%)</u>
80S	70	30
60S	64	36
40S	65	35

The ratio of absorbances at 260 nm to 280 nm and chemical analysis data of RNA and protein content indicate that the two isolated ribosome subunits have similar protein and RNA contents. Tissieres et al. (1959) reported the composition of E. coli 70S ribosomes and 50S and 30S subunits to be the same, whereas Friedman et al. (1966) reported different composition between two subunits of R. spheroids. The composition of the 40S and 60S subunits are different from the 80S ribosomes. The subunits have lower RNA content than the 80S particle. The elution pattern of subunits (Figure 24) showed, in addition to two subunit peaks, a slowly moving peak near the top. This peak has been observed in other systems also (see, for example, Hamilton and Ruth, 1967). Analysis of the peak by disc gel electrophoresis did not give free protein bands, and the ultraviolet spectrum had maximum peak at 258 nm.

Properties of r-proteins from 1M LiCl split free protein fraction and particulate fraction

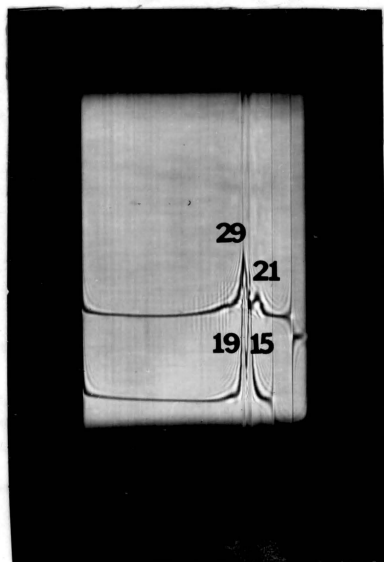
Figure 27 shows the sedimentation pattern of 1M LiCl treated ribosomes dialyzed against a medium containing  $1.5 \times 10^{-3}M$   $MgCl_2$ , 0.025M Tris-HCl (pH 7.8), and 0.020M KCl. The sedimentation pattern showed two peaks with  $S_{20,w} = 21S$  and 29S. When  $MgCl_2$  in the dialyzate buffer was replaced by  $1 \times 10^{-4}M$  EDTA, the pattern had two peaks with  $S_{20,w}$  of 19S and 15S, respectively. When the  $C^{14}$ -DNS-ribosome was separated into free protein and particulate fraction by 1M LiCl treatment the yield

Figure 27. Analytical ultracentrifuge patterns of the 1M LiCl treated ribosomes dialyzed vs. 0.025M Tris-HCl (pH 7.8), 0.020M KCl, 0.006M 2-mercaptoethanol, and magnesium concentration of desired as indicated below. Schlieren patterns of control and dansylated ribosomes were taken at a phase angle of  $50^\circ$  and 25 minutes after the centrifuge reached 38,362 rpm at  $4.4^\circ$ . Sedimentation was from right to left.

Top:  $1.5 \times 10^{-3} \text{M}$   $\text{MgCl}_2$

Bottom:  $1 \times 10^{-4} \text{M}$  EDTA

Numbers in figure are sedimentation coefficients in svedberg unit ( $s_{20,w}$ ).



of r-protein of the former was 1.7 times that of the latter. However, the radioactivity per gram of protein, which may be the measure of the degree of dansylation, from the particulate fraction was 4.4 times that of the free protein fraction.

#### Infra-red spectra of pine seed ribosomes

Since one objective of this work was to investigate any variance in composition of minor components in ribosomal species during successive washing procedures, high concentration of samples (3 mg sample per 100 mg KBr) was used to enhance weak spectrum patterns of small amount of contaminants. The absorbance peak at  $960\text{ cm}^{-1}$ , which is characteristic for RNA and quantitatively proportional to the amount of RNA, increased as the  $B_1$ -ribosome was washed successively as described in the preparation of ribosomes. Thus, this observation strengthens the findings that proteins are detached by washing process, thereby increasing the RNA content (Figure 28). In order to investigate the nature of the RNA-protein binding in the ribosome and the presence of putative minor components, such as lipids, in the ribosome, the infrared spectra of the mixture of extracted r-RNA and r-protein were compared with that of ribosomes. No significant (qualitative) differences between the two spectra could be detected (Figure 29). Further investigations along this line compare the infra-red spectra of  $B_1$ -ribosome,  $NH_4$ -ribosomes, and  $B_2$ -ribosomes (Figure 29 and Figure 30). Except for the band intensity of  $960\text{ cm}^{-1}$ , significant differences could not be detected. In the present work, the difference spectra technique was not employed, although such techniques may have been more useful. Infrared spectra of Figure 29 and 30 were chiefly for the study of weak bands of putative minor

Figure 28. Infrared spectra of (1) B<sub>1</sub>-ribosome, (2) NH<sub>4</sub>-ribosome, and (3) B<sub>2</sub>-ribosome. Five mg portion of a mixture of 3 mg sample and 100 mg KBr powder was used in making a micro pellet of KBr.



PER CENT TRANSMISSION —↑

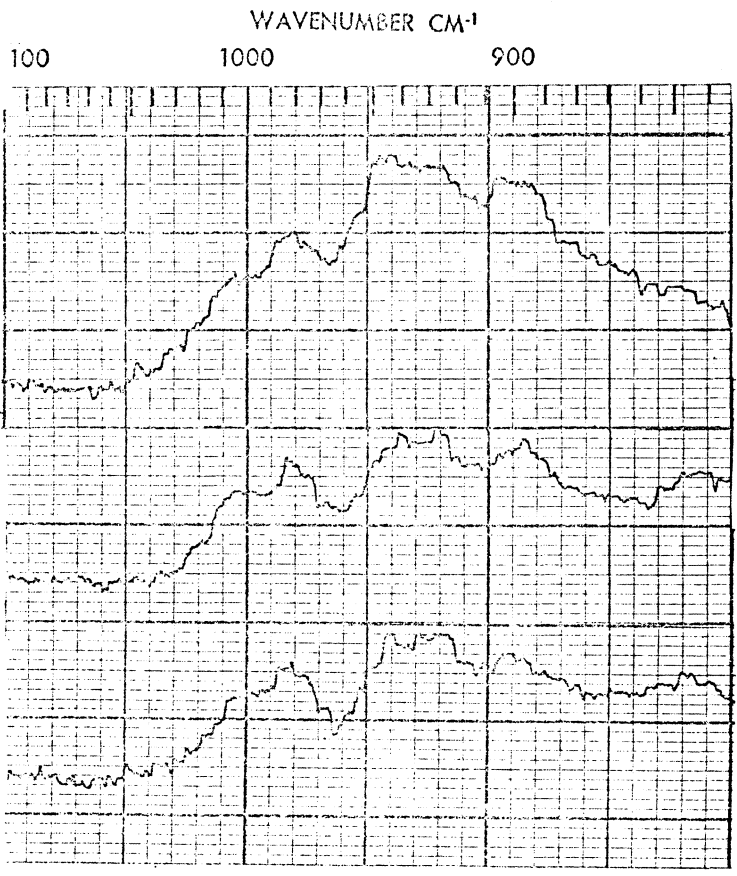


Figure 29. Infrared spectra of B<sub>2</sub>-ribosome (top picture) and that of a mixture of r-RNA and r-protein (bottom picture).

Top picture: 3 mg of lyophilized and pulverized B<sub>2</sub>-ribosome pellet were mixed with 100 mg KBr powder, and then 4 mg portion of the mixture was pressed into a micro pellet.

Bottom picture: 1.43 mg RNA, 1.43 mg r-protein, and 0.15 mg of lyophilized buffer B residue were mixed with 100 mg of KBr, and then 4 mg portion of the mixture was pressed into a micro pellet.

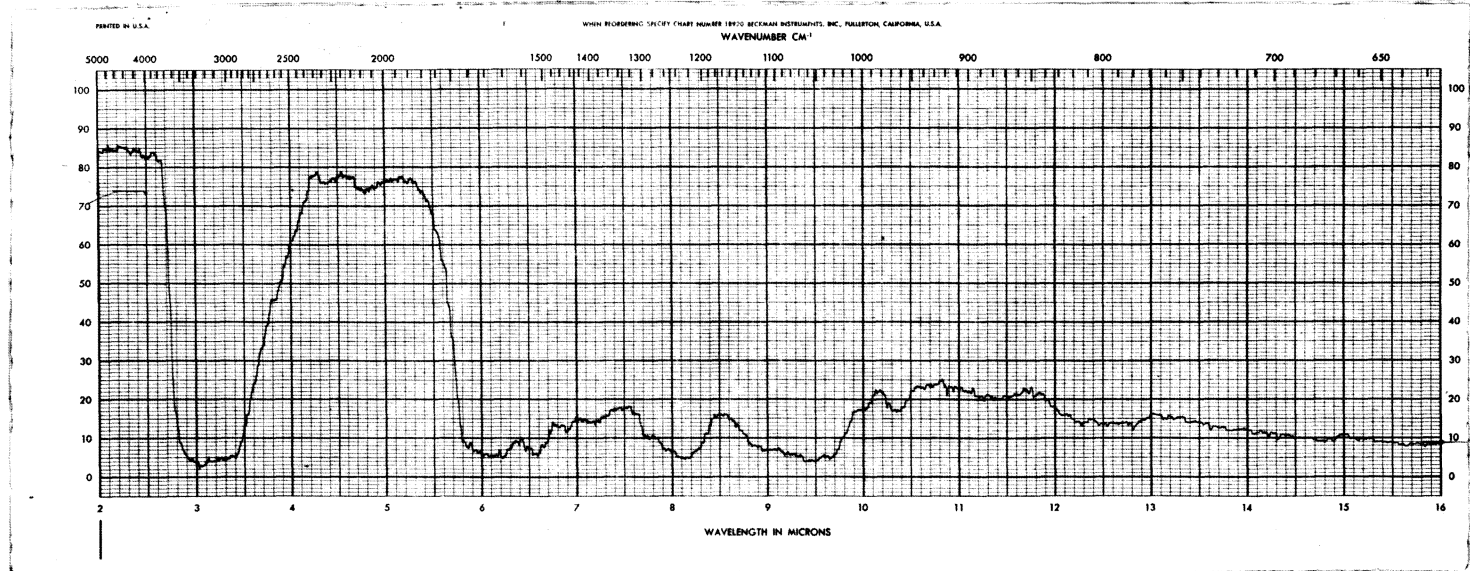
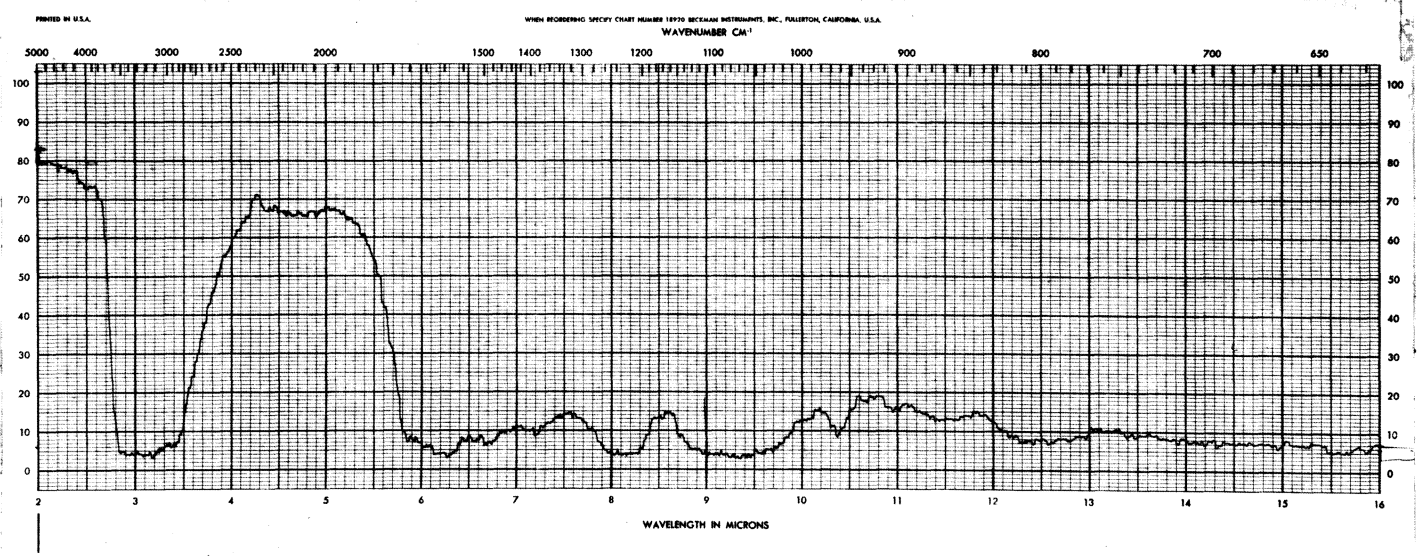
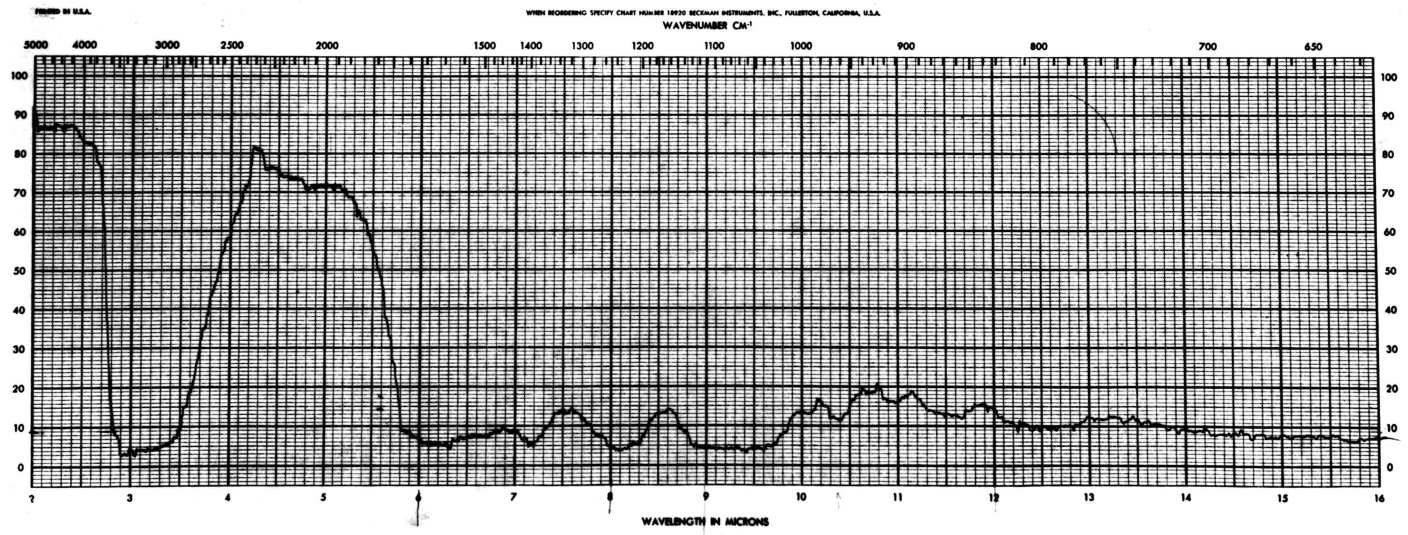
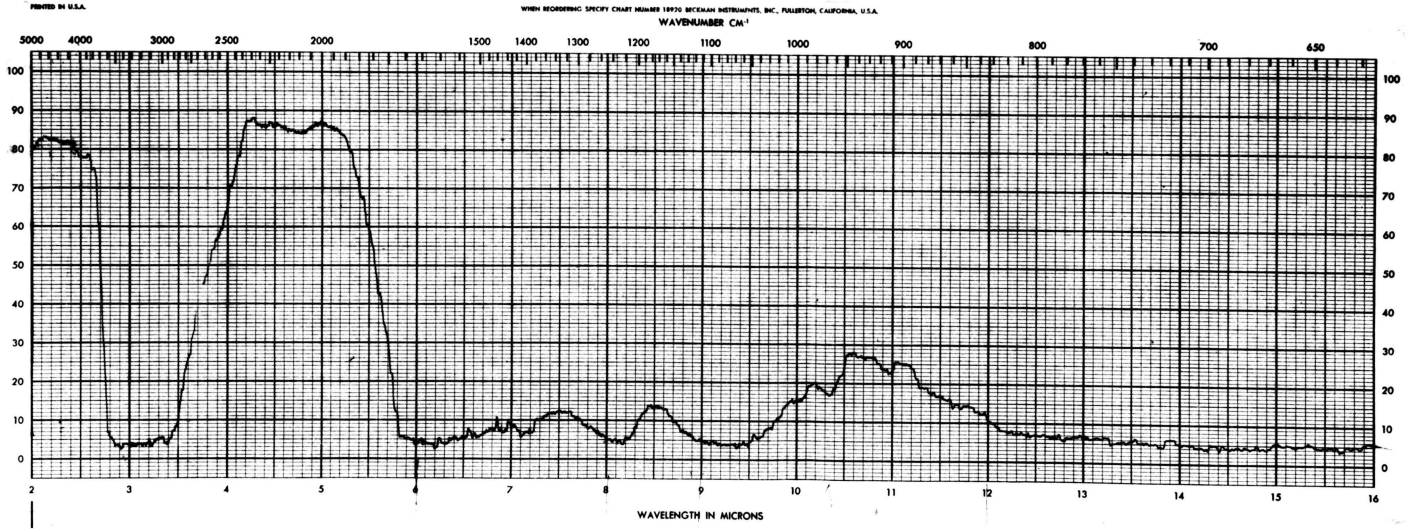


Figure 30. Infrared spectra of B<sub>1</sub>-ribosome (top picture) and NH<sub>4</sub>-ribosome (bottom picture). The method of micro pellet preparation of these samples was same as that for B<sub>2</sub>-ribosome in Figure 29.

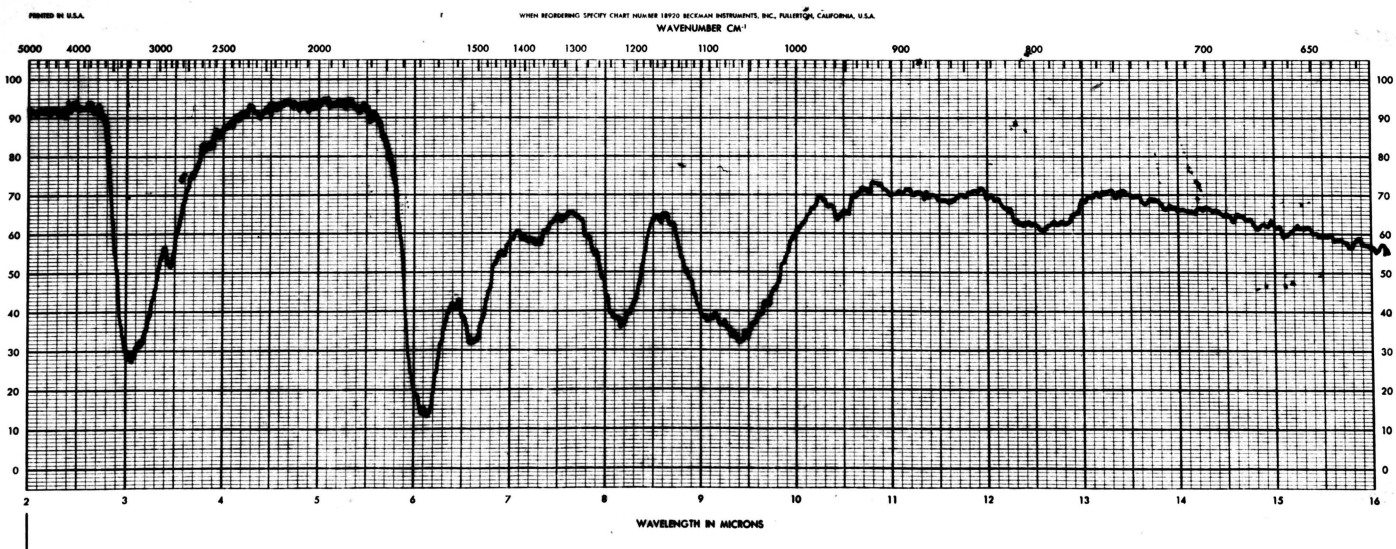
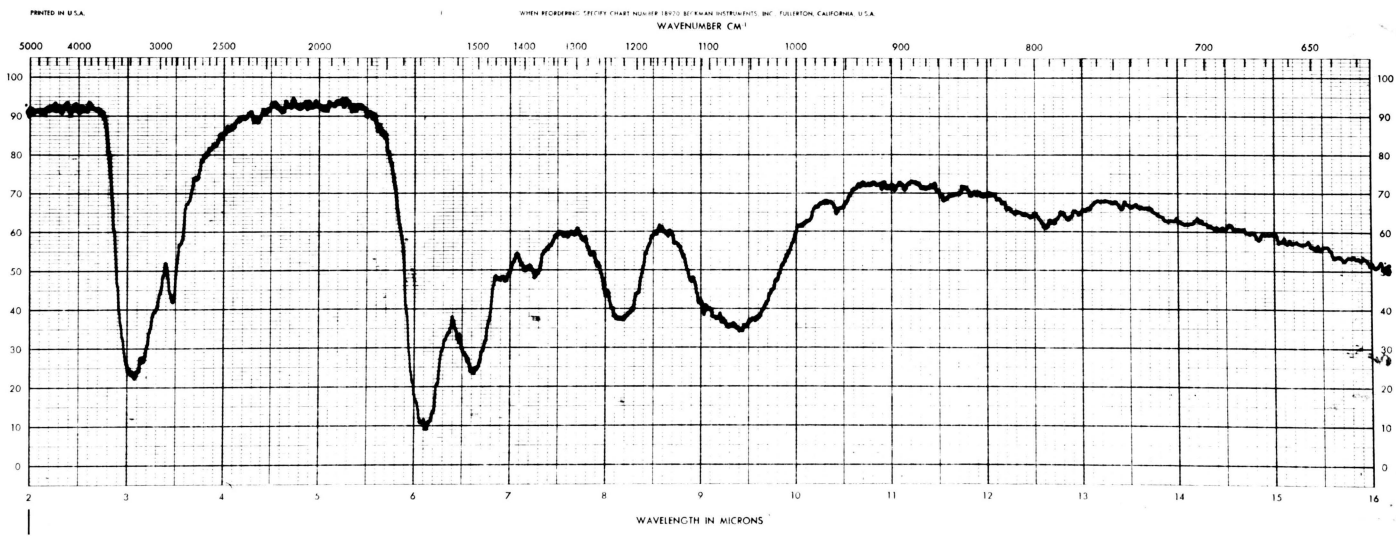


components in the ribosomes. Figure 31 shows the infrared spectra of the  $\text{NH}_4$ -ribosome and the  $\text{B}_2$ -ribosome with decreased sample concentrations. They show more clearly the stronger bands of the major components, RNA and protein. The infrared spectra of  $\text{NH}_4$ -ribosomes and  $\text{B}_2$ -ribosomes are also similar. In the infrared spectra of ribosomes, a weak band near  $3080\text{ cm}^{-1}$  due to NH absorption and characteristic for protein has disappeared. This might be due to either hydrogen bond formation of this group with the RNA component or masking by bands of RNA. Other bands characteristic for protein were preserved in the spectrum of ribosomes: a band at  $3300\text{ cm}^{-1}$  due to hydrogen bonded NH groups, and amide I and amide II bands in the  $1600\text{--}1500\text{ cm}^{-1}$  region. The bands in the infrared spectrum of the ribosome contributed mainly by RNA components are: groups of bands in  $1800\text{--}1500\text{ cm}^{-1}$  regions, which may be overlapped with protein bands; a strong band at  $1220\text{ cm}^{-1}$  due to phosphate stretching vibration, groups of strong bands in the  $1100\text{--}1000\text{ cm}^{-1}$  region assigned to the  $\text{PO}_2^-$  stretching vibration and the C-O stretching vibrations of ribose. A weak band at  $790\text{ cm}^{-1}$  may be due to P-O stretching, C-O stretching, and NH out-of-plane bending vibrations.

#### Characteristics of extracted r-proteins

**Physicochemical heterogeneity:** Electrophoretic and isoelectric charge heterogeneity of r-proteins from 80S ribosomes, and from 60S and 40S subunit ribosomes have been discussed previously. As another criterion for such heterogeneity, analytical ultracentrifuge sedimentation patterns of r-proteins were tested. The analytical ultracentrifuge pattern of r-proteins dissolved in 8M urea (pH 4.7), 0.15M KCl, 0.05M

Figure 31. Infrared spectra of  $\text{NH}_4$ -ribosome (top) and  $\text{B}_2$ -ribosome (bottom). Methods of the preparation of micro pellets were same as those in Figure 29 except that 1.5 mg, instead of 3 mg were mixed with 100 mg KBr.

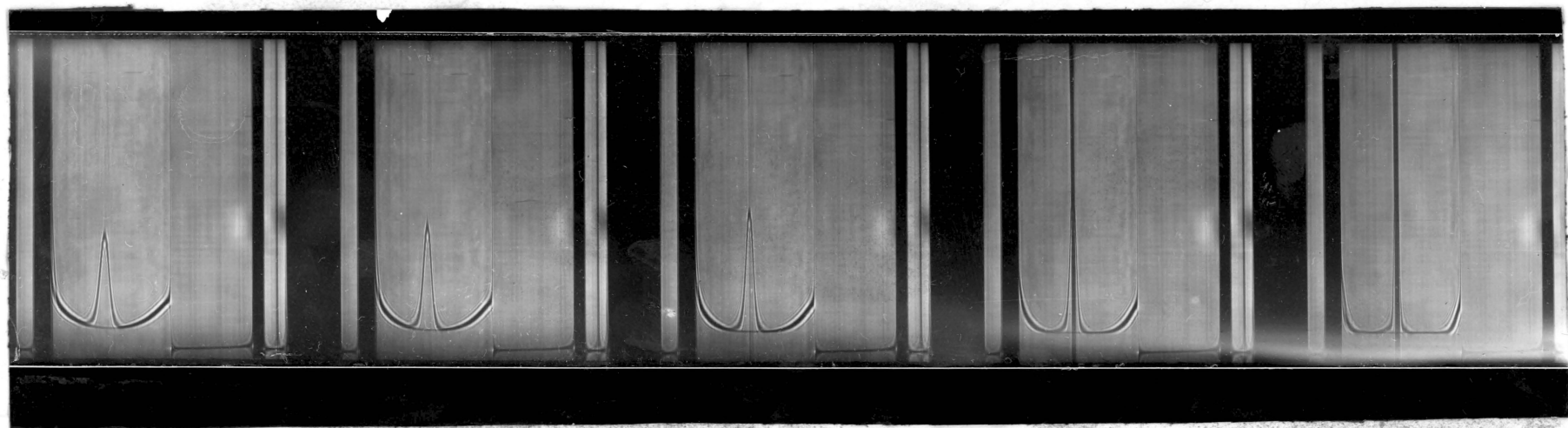




acetic acid, 0.02M sodium acetate, and either 0.006M 2-mercaptoethanol or 0.005M DTT (dithiothreitol) had a monodispersed pattern ( $s_{20,w}=0.4S$ , Figure 32), whereas in the medium with either reducing agent omitted, a small fast moving shoulder was observed ( $s_{20,w}=0.5S$ , Figure 33). Similar patterns in the E. coli system with reducing agent omitted have been reported by Möller and Chrambach (1967), Wolfe and Kay (1968) reported that without urea and DTT in the solvent, the ultracentrifuge patterns indicated a marked polydispersity of the r-proteins. They suggested that such aggregation might be formed by disulfide bridge formation between r-protein components. Petermann (1964b) suggested that high content of basic and acidic amino acids in r-proteins might lead to salt formation and aggregation. The dependence of the sedimentation coefficient ( $s_{20,w}$ ) of r-proteins on total protein concentration is shown in Figure 34. The extrapolated value of  $s_{20,w}$  to zero concentration ( $s_{20,w}$ ) was 1.0S, which is similar to other systems reported (0.9S from buds of pea seedlings, Setterfield, 1960; 1.3S from wheat embryo, Wolfe and Kay, 1968). The  $\bar{M}_w$  measured by the sedimentation-equilibrium technique was  $13,000 \pm 1000$ .

Ultraviolet and infrared spectra: Ultraviolet and infrared spectra of r-proteins are shown in Figure 35 and Figure 36, respectively. The ultraviolet spectrum of r-protein in 8M urea (pH 4.7), 0.15M KCl, 0.05M acetic acid, 0.02M sodium acetate, and 0.006M 2-mercaptoethanol had a maximum absorbance peak at wavelength 277 nm and minimum at 251 nm. The infrared spectrum of r-protein exhibited absorbance peaks at the  $3300\text{cm}^{-1}$  region of hydrogen-bonded NH groups, and in the region of  $1600\text{--}1500\text{cm}^{-1}$  amide I and amide II bands, and a weak band near  $3080\text{cm}^{-1}$ , due to

Figure 32. Analytical ultracentrifuge patterns of r-proteins (1%) in 8M urea (pH 4.7), 0.15M KCl, 0.05M acetic acid, 0.02M sodium acetate, and 0.005M DTT (dithiothreitol). Schlieren patterns were taken at a phase angle of  $70^\circ$  and 1, 9, 17, 25 and 33 minutes after the centrifuge reached 40,657 rpm at  $8.0^\circ$ . Sedimentation was from right to left.



33

25

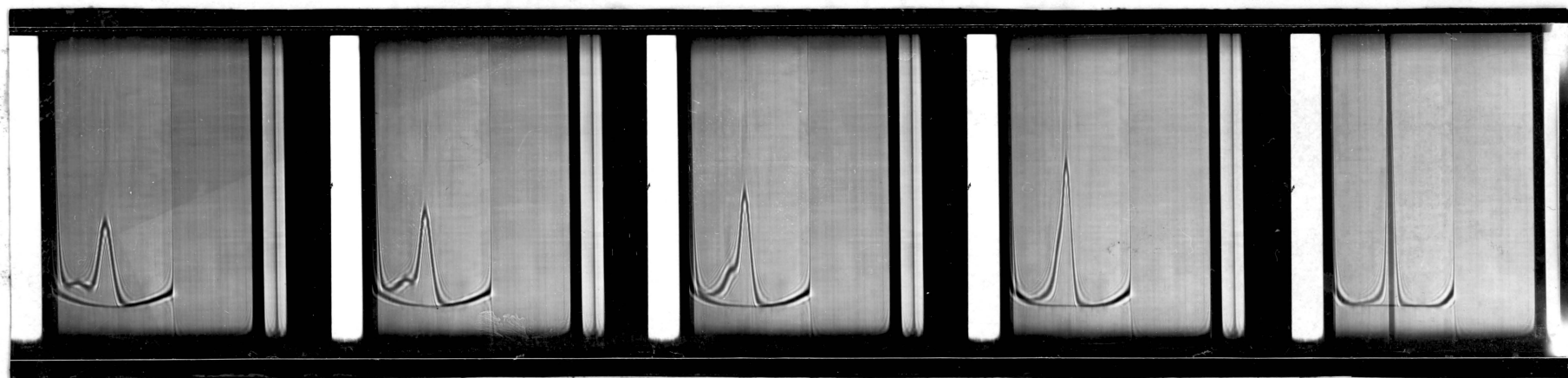
17

9

1

MIN.

Figure 33. Analytical ultracentrifuge patterns of r-proteins (1%) in 8M urea (pH 4.7), 0.15M KCl, 0.05M acetic acid, 0.02M sodium acetate. Schlieren patterns were taken at a phase angle of  $60^\circ$  and 1, 9, 17, 25, and 33 minutes after the centrifuge reached 56,100 rpm at  $8.0^\circ$ . Sedimentation was from right to left.



33

25

17

9

1

MIN.

Figure 34. The concentration dependence of the sedimentation coefficients of r-proteins. Ribosomal proteins were dissolved in 8M urea (pH 4.7), 0.15M KCl, 0.05M acetic acid, 0.02M sodium acetate and 0.006M 2-mercaptoethanol. The concentration was based on the weight of r-proteins lyophilized.

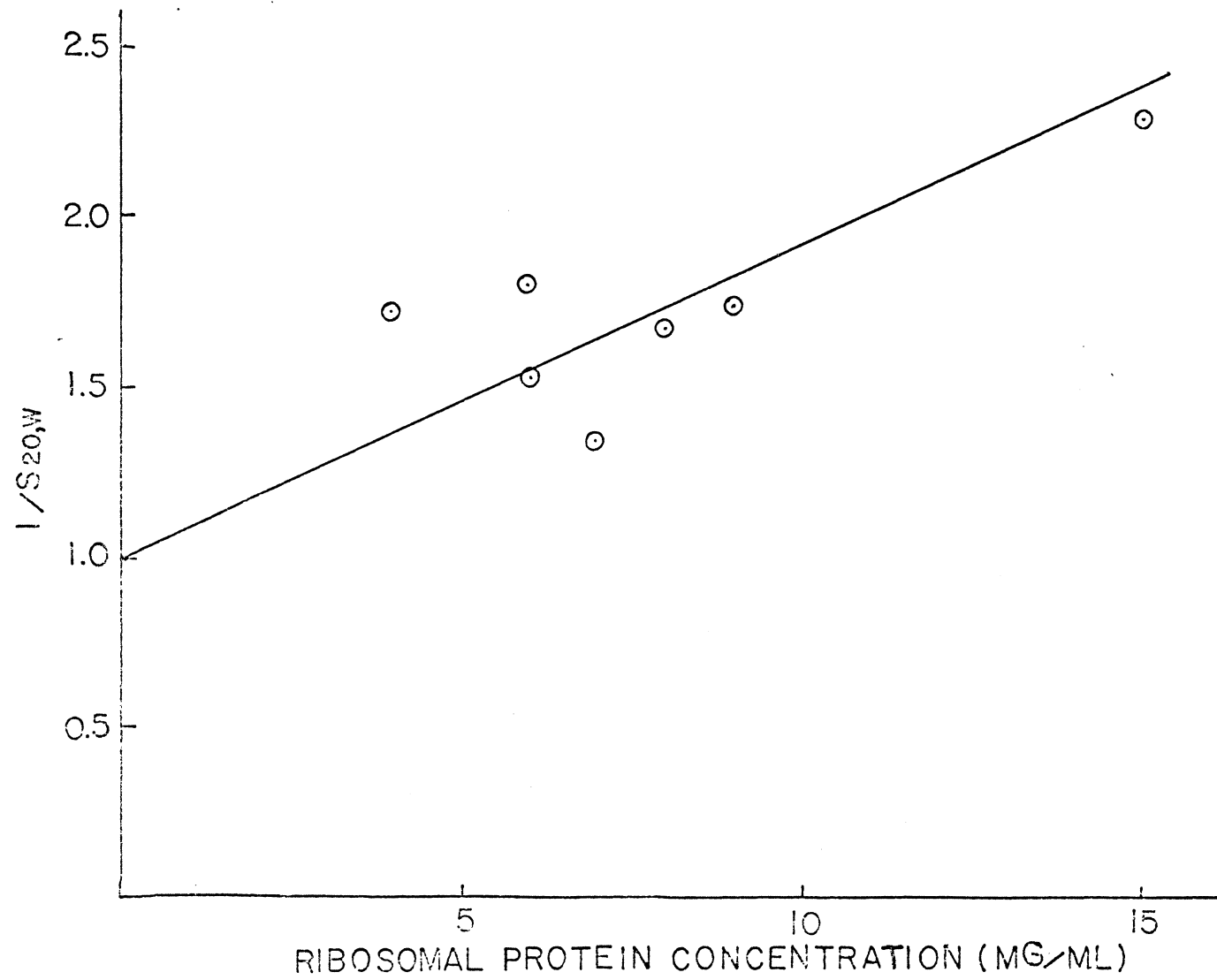


Figure 35. Ultraviolet spectrum of r-proteins in 8M urea (pH 4.7), 0.15M KCl, 0.05M acetic acid, 0.02M sodium acetate, 0.006M mercaptoethanol.



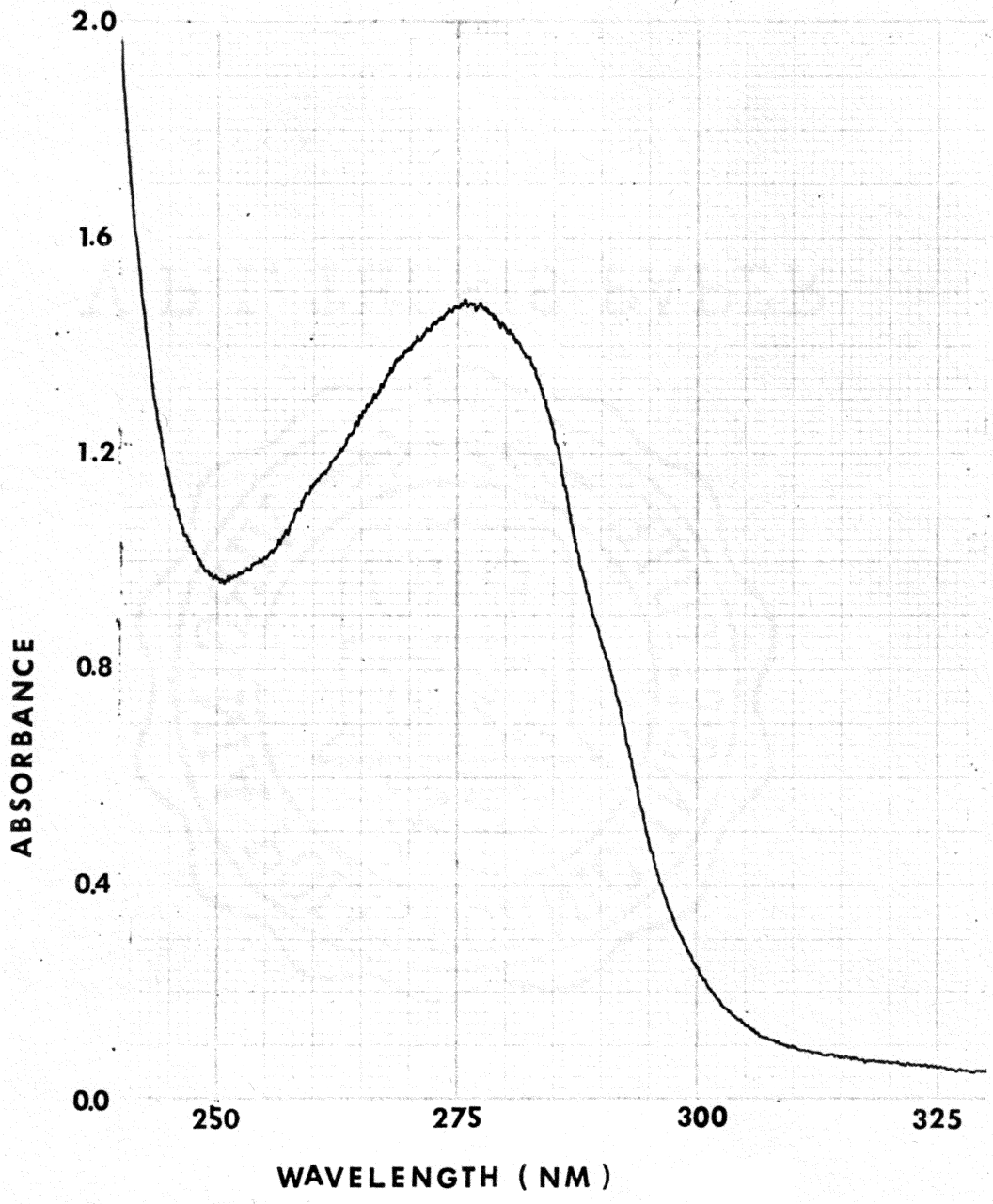
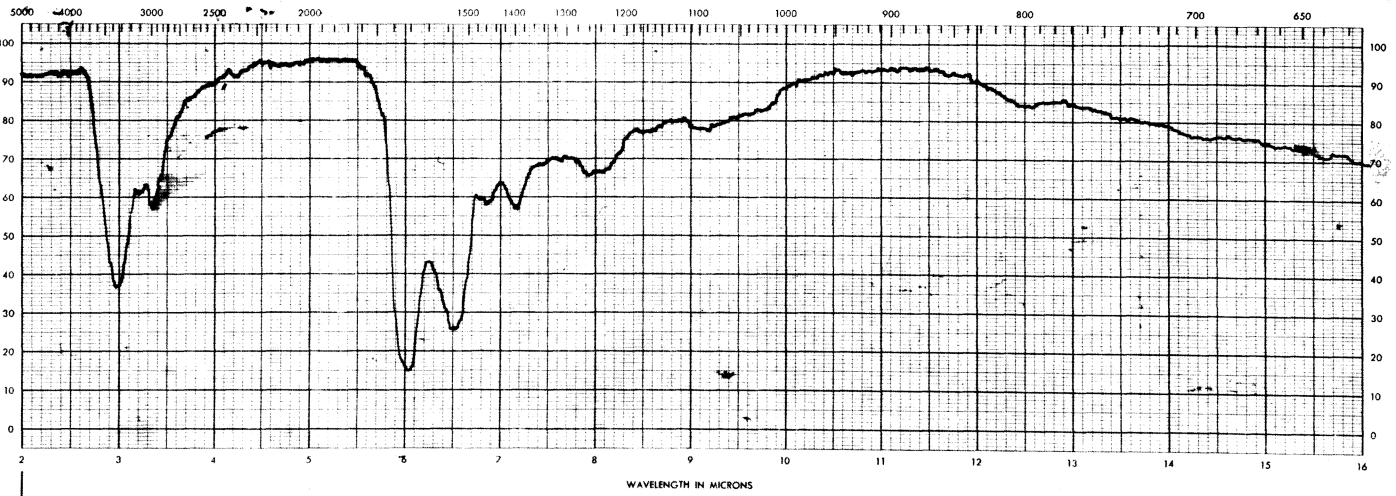


Figure 36. Infrared spectrum of r-proteins. A 4 mg portion of a mixture of 1.5 mg of protein and 100 mg KBr powder was pressed into a micro pellet. Detailed methods are described in Materials and Methods.

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additional NH absorption. All of these are characteristic patterns of proteins. However, the infrared spectrum of the r-proteins was free of the  $960\text{ cm}^{-1}$  band, which is characteristic for RNA. The recovery of r-proteins by the acetic acid method was 86-91% of the total r-protein, whereas in LiCl-urea method more than 98% of the total protein of ribosomes was recovered.

Amino acid composition: The amino acid composition of pine seed r-proteins, in mole percent, is listed in Table IV. The tryptophan and cysteine content of the proteins was not measured. Correction for the destructive hydrolysis of serine, methionine, and threonine or for the relatively slow liberation of leucine, isoleucine, and valine from peptides was not made. The amino acid composition of pine seed r-proteins was very similar to those of other species in that it had high content of both basic and acidic amino acids, a low amount of aromatic and sulfur containing amino acids, and the ratio of basic amino acids to acidic amino acids was approximately 1. In Table V, amino acid compositions of the proteins from the supernatant fractions and B<sub>2</sub>-ribosomes are shown. Analysis patterns of valine were irregular and were omitted from the table. It is noteworthy that proteins from the supernatant fractions (B<sub>1</sub>S, NH<sub>4</sub>S, and B<sub>2</sub>S) contained higher acidic amino acids and higher ammonia content than that from the B<sub>2</sub>-ribosomes (B<sub>2</sub>R). Hardy et al. (1969) also reported that proteins, which were removed from E. coli ribosomes by ammonium sulfate, were a heterogeneous population of proteins which were more acidic than most of the remaining r-proteins. Furthermore, the ratio of the content of ammonia to acidic amino acids of the proteins from the supernatant fractions and the proteins from the B<sub>2</sub>-ribosomes were similar,

Table IV Amino acid compositions of r-proteins (in mole percent)

Amino acid	Source				
	Pine seed	Wheat embryo <sup>a</sup>	Pea seed-lings <sup>b</sup>	E. coli <sup>c</sup>	Rat liver <sup>d</sup>
Lysine	11.1	9.1	9.1	9.01	9.63
Histidine	2.3	1.8	1.4	1.91	2.38
Arginine	8.1	8.2	6.3	7.30	7.53
Aspartic acid	8.2	8.4	9.5	8.30	8.06
Threonine	4.2	4.9	5.6	5.52	5.06
Serine	5.0	4.2	5.9	4.38	4.85
Glutamic acid	10.3	11.1	10.2	10.08	8.87
Proline	5.1	3.1	5.0	3.67	4.93
Glycine	9.0	8.6	8.1	8.18	7.72
Alanine	7.6	11.8	8.4	10.98	8.58
Half-cystine	+	-	-	0.53	1.09
Valine	8.2	8.9	7.4	9.63	7.98
Methionine	-	2.2	1.6	2.40	2.14
Isoleucine	4.6	5.3	5.7	5.51	4.92
Leucine	7.7	7.5	8.7	7.40	8.78
Tyrosine	2.1	1.9	3.0	1.78	2.93
Phenylalanine	3.0	3.1	4.1	3.03	3.80
Basic	21.5	20.8	16.8	18.22	19.54
Acidic	18.5	18.	19.7	18.38	16.93
Basic/acidic	1.16	1.15	0.85	0.99	1.15

<sup>a</sup> Wolfe and Kay, 1968.

<sup>c</sup> Spah, 1962.

<sup>b</sup> Birnstiel *et al.*, 1964.

<sup>d</sup> Crampton and Petermann, 1959.

Table V Amino acid composition (in mole percent) of proteins from supernatants and the B<sub>2</sub>-ribosome, split and particulate fraction

Amino acid	Source of Protein					
	B <sub>2</sub> R	B <sub>1</sub> S	NH <sub>4</sub> S	B <sub>2</sub> S	Split	Particulate
Lysine	11.1	6.8	6.8	9.0	14.1	12.1
Histidine	2.3	3.4	3.3	2.3	2.8	3.0
Arginine	8.1	7.6	12.9	7.1	10.3	8.8
Aspartic acid	8.2	11.5	10.4	9.8	9.4	9.6
Threonine	4.2	5.3	3.5	5.2	4.7	5.1
Serine	5.0	5.8	6.0	5.8	5.1	5.5
Glutamic acid	10.3	16.1	18.8	12.3	10.4	10.2
Proline	5.1	5.8	5.5	5.3	5.2	4.6
Glycine	9.0	10.4	8.6	11.6	9.8	10.6
Alanine	7.6	8.3	8.3	12.1	9.4	9.6
Half cystine	+	+	+	+	+	+
Methionine	-	-	-	-	-	-
Isoleucine	4.6	5.2	3.7	5.4	5.1	6.0
Leucine	7.7	7.8	7.0	8.0	7.8	9.0
Tyrosine	2.1	2.4	2.2	2.3	2.6	2.6
Phenylalanine	3.0	3.5	2.8	3.4	3.3	3.4
Ammonia	10.29	14.88	16.16	13.64	11.76	13.04
Basic amino acids	21.5	17.8	16.2	18.4	27.2	23.9
Acidic amino acids	18.5	27.6	29.2	22.1	19.8	19.8
Basic/acidic	1.16	0.64	0.55	0.83	1.37	1.20
Ammonia/acidic	0.55	0.53	0.55	0.61	0.59	0.65

i.e. about 0.56. In the section on isoelectric focusing, it was shown that proteins, which were more readily detachable into the wash supernatants, were mainly neutral proteins (pI range from 6.3 to 7.5), while the proteins remaining in the ribosome contained mostly basic proteins. This observation is compatible with the above described amino analysis data. A high percent of acidic amino acids may exist in ribosomes mostly as amide forms. In Table V, the amino acid compositions of split and core proteins are very similar in that they have higher basic amino acid content than acidic amino acid content. Some workers (Spahr, 1962; Atsmon et al., 1966; Traub et al., 1966) reported that the particle fraction was much more basic than the total ribosomal protein, whereas the released fraction was correspondingly less basic, being in fact, nearly neutral, as shown by amino acid analysis. It appears that their ribosome samples, from which released and particle fractions were derived, might have been washed less extensively than in the present work.

Amino-terminal amino acid determination: Alanine, arginine, and lysine (least among the three) were the main  $\text{NH}_2$ -terminal amino acids. It was possible that serine, threonine, valine, leucine, and/or isoleucine were  $\text{NH}_2$ -terminal amino acids but this could not be confirmed in the present investigation. Present work was limited only to qualitative determination. The  $\text{NH}_2$ -terminal group patterns are considerably different, depending on the source of the r-protein. As the  $\text{NH}_2$ -terminal group, alanine was detected in all species as the main component (E. coli, Waller and Harris, 1961; yeast, Haruna, 1963; rat liver, Cohn and Simpson, 1963; reticulocytes, Mathias and Williamson, 1964; wheat embryo,

Wolfe and Kay, 1968; pea seedlings, Setterfield et al. (1960). Lysine was detected as an NH<sub>2</sub>-terminal amino acid in small amounts in the r-protein from pea seedlings (Setterfield, et al., 1960) and E. coli (Waller and Harris, 1961). Arginine has not been detected in any species so far reported. All works published have been done using the dinitrophenyl amino acid method. Methionine has been reported to be the main NH<sub>2</sub>-terminal amino acid in wheat embryo (Wolfe and Kay, 1968), in E. coli (Moore et al., 1968 and Waller and Harris, 1961) and in reticulocytes (Mathias and Williamson, 1964). Amino-terminal amino acids of valine, glycine, serine, threonine, leucine, lysine, glutamic acid, aspartic acid, and proline were found in certain species.



## V. DISCUSSION

### Possible properties and organization of r-proteins as a structural element in the ribosome

As discussed previously, an understanding of the minor protein components in the ribosomes and the detachability of r-proteins from the ribosome without causing the destruction of ribosomal integrity appear to be important factors for understanding the nature of the entity of the r-proteins in the ribosome. In fact, most workers are primarily interested in investigations of these proteins remaining in the ribosome after purification, ignoring those proteins which have been removed from ribosomes. Investigations of this type led to the proposal that every r-protein of the 30S subunit was present in a single copy (Moore et al., 1968; Traut et al., 1970; Sypherd et al., 1970). Hill et al. (1969) suggested the homogeneity of E. coli ribosomes based on sedimentation-equilibrium studies with ribosomes, which had been extensively washed, disregarding the nature and pattern of those proteins removed in their washing procedure. In their proposal for the existence of r-protein from the 30S subunit of E. coli ribosomes in multiple copies and the heterogeneity of E. coli 30S ribosomes, Kurland et al. (1970) were not certain whether or not they had removed any r-proteins during purification of the particles. Present work with pine seeds demonstrated that isoelectric focusing patterns of removed proteins overlapped with those remaining in the ribosomes, and that the detachability was different, depending upon the pI's of the proteins. Therefore, presumably, the pairs of overlapping proteins, removed and remaining, might consist of identical protein components, although definitive conclusion awaits

the proof of identical primary structures of the overlapping pairs of proteins. The difference in the amino acid composition between the removed proteins and the proteins remaining in the ribosomes may be due to the difference in detachability of the proteins in the ribosomes, that is, proteins in the ribosome are not removed to the same extent. The higher content of basic amino acids in the remaining proteins may reflect the observation that the most basic proteins were the least detachable. Hardy et al. (1969, Kurland group) analyzed the proteins which had been washed off the crude ribosomes by ammonium sulfate and found that they were chromatographically (phosphocellulose) and electrophoretically (disc gel) distinguishable from the proteins which remained on the ribosome. This was confirmed in the present work using disc gel electrophoresis (it is not included in this dissertation and will be published elsewhere). These observations seemingly conflict with those made by isoelectric focusing, since isoelectric focusing patterns of removed proteins overlap with those remaining in the ribosomes after washing. As mentioned in the "Introduction" the migration patterns of proteins in chromatography or disc gel electrophoresis are affected by several factors such as size, charge, and conformation. In contrast, the migration patterns of proteins in isoelectric focusing are dependent upon the pI's of proteins and are free from size and conformation. Therefore it can be speculated that the size and shape of overlapping pairs of proteins are distinguishable from each other, whereas their pI's are same. If the primary structures of the overlapping pairs of proteins are identical, it might be concluded that r-proteins are composed of multiple copies of fundamental subunit proteins. In such

a case, each r-protein component band in the isoelectric focusing pattern would represent a kind of fundamental subunit, and the number of elementary protein units might be equal to the number of the protein component bands isolated by isoelectric focusing. The observation that r-protein bands focused in isoelectric focusing are distributed in a few discrete groups and the detachability of such group proteins is related with their pI values, is interesting in connection with other worker's reports that there is a definite order of r-protein association with r-RNA in reconstitution of the ribosome from r-protein and r-RNA (Traub and Nomura, 1970b). Apparently, r-proteins add on in groups, forming only a few discrete kinds of precursor particles rather than a continuous spectrum (Britten et al., 1962). The observation that proteins in a particulate fraction were 44 times more dansylated than the free protein fraction, although their amino acid compositions and isoelectric focusing patterns were similar, might indicate that physicochemical properties of r-proteins in ribosomes might be different depending upon their positions in the ribosome.

#### Some possible functions of r-proteins in ribosomes

The failure to observe any difference between the normal ribosomes and the DNS-ribosomes in their dissociation or reassociation patterns as examined by sedimentation analysis and by the temperature-absorbance profiles (260 nm) below 36° may lead to the speculation that dansylation of the ribosome did not affect its conformation under these conditions. However, there was a difference in the T<sub>m</sub>'s between normal and DNS-ribosomes, that of the DNS-ribosome being 2° lower than that of the normal and the slope of the differentiated melting curve of the DNS-ribosome

at the  $T_m$  was 0.057 greater than that of the normal ribosome. The melting temperature of a r-RNA is directly proportional and the slope of the differentiated melting curve is reciprocally proportional to double helical content (Cotter et al., 1967; Cox, 1970). The absorption at 260 nm by r-protein was observed to remain unchanged with increasing temperature (Cotter et al., 1967). Therefore, it might be speculated that the dansylation of the ribosome accelerated the thermal conformational change of the double helical structure of r-RNA in ribosomes into the single stranded form. This would mean that ribosomal protein might have some function in the maintenance of the double helical configuration of r-RNA in the ribosome, and that dansylation of the r-protein weakened the function. Dansyl chloride reacts with basic groups in proteins, such as primary and secondary amines, phenolic hydroxyls, thiols, and imidazoles. A single dansylation decreases the net charge of a protein molecule by one. The amino groups of the protein, mostly in the  $-NH_3^+$  form, will lose their positive charge after reacting with the dansyl chloride (DNS-Cl) to form the corresponding derivative. However the stabilization of the double-helical structure of RNA is due largely to the decrease in electrostatic repulsion between the negatively charged phosphate groups of RNA by charge shielding. If we assume that proteins are important for the maintenance of the double helical structure, a decrease of positive charges of the r-protein by dansylation may lead to decreased charge shielding, increased electrostatic repulsion of negatively charged phosphate groups of the helix, and consequently less stabilization of the double helical strand.

The 40S ribosomal subunit is more sensitive to ribonuclease than the 60S subunit at higher ( $5 \times 10^{-3} \text{M}$ ) magnesium concentration (Figure 11). However, as magnesium concentration is reduced to the level of  $1 \times 10^{-4} \text{M}$ , this difference in ribonuclease sensitivity becomes smaller. The higher sensitivity of the smaller subunit has been reported by others, who observed that the smaller subunit could be protected from ribonuclease degradation by association with the larger subunit (Friedman et al., 1966; Hess and Horn, 1964; Worcel et al., 1968). Based on their electron microscopic observations, Worcel et al. proposed a model which has the RNA moiety as an inner core of the ribosome. The present work does not seem to support this model. If the RNA moiety forms an inner core of the ribosome, the ribonuclease sensitivity of the DNS-ribosome and the normal ribosome should be similar at high magnesium concentrations. However, in the present work, DNS-ribosomes exhibit higher ribonuclease sensitivity at both high and low magnesium concentration levels than normal ribosomes. Therefore, we can speculate that protein might be an important factor for the ribonuclease sensitivity of RNA in the ribosome. Ribosomal RNA might be protected by the r-protein whether the ribosome is folded or unfolded. Dansylation of r-protein may affect the protection of RNA by r-protein against ribonuclease degradation. However, the dansylation effect on the protection of RNA by r-protein might not be due to conformational change of r-RNA, since normal ribosomes and DNS-ribosomes have similar conformation based on the sedimentation patterns and the temperature-absorbance profiles at low temperatures. Evidences that r-proteins are substantially associated with the nonhelical portions of the RNA has been discussed in the introduction. It

should be remembered that nonhelical single-stranded portions of RNA are sensitive to ribonuclease degradation, whereas double-stranded portions are resistant. Therefore, it might be concluded that the ribonuclease sensitive single-stranded portions of r-RNA might be protected against ribonuclease by the r-proteins associated with the region. Cotter et al. (1967) reported a ribosomal model in which the surface of the ribosome consisted chiefly of double helical RNA projecting outwards from the surface and the proteins were in the interior and associated with nonhelical regions of the RNA. However, Cox (1969) reported that high concentration ( $>0.36\mu\text{g}$  of RNase/mg of RNA) of RNase can readily hydrolyze exposed double helical regions of r-RNA of the ribosome, while within the ribosome double helical secondary structure survives exposure to high concentrations of ribonuclease, and even after the RNA moiety is converted into a single-stranded form, its sensitivity towards ribonuclease is not substantially increased. The present work cannot explain how double stranded r-RNA inside the ribosome can be more resistant to ribonuclease than that of the surface. We can only imagine that r-proteins associated with RNA may interact with each other to form a protein matrix, which might inhibit the approach of ribonuclease to the double helical region of the RNA.

Many workers suggested that the binding of the r-protein and the r-RNA neither involve ionic (Traub and Nomura, 1970) nor covalent bonds (Spitnik-Elson and Atsmon, 1969). In the present work, comparing the infrared spectra of the ribosome and a mixture of the r-RNA and the r-protein, any clue for such bonding was not detected. However, the difference in the detachability of r-proteins from ribosomes, depending

upon their pI's, may indicate possible differences in the strength of binding between the r-RNA and the r-protein according to the species of r-protein components.

#### Usefulness of DNS-ribosomes in the study of the function of r-proteins in protein synthesis

In the present work, the functional role of r-proteins in protein synthesis processes by ribosomes were not investigated. However, the results of the present work demonstrate that DNS-ribosomes can be a noble tool in studying the function, since the dansylation of ribosomes can be performed effectively without affecting the integrity of the ribosome. At present, our knowledge about any function of r-protein in specific biosynthetic process is at a rudimentary stage. Progress in the area will require development of techniques to study r-protein function. The DNS-ribosome technique of the present work may be such a technique.

#### Characteristics of r-Proteins

The disc gel electrophoresis pattern showed approximately 25 bands. If we assume that these 25 bands represented total unique r-protein components, then the total  $\bar{M}_w$  of r-proteins per pine seed ribosomal particle would be about  $13,200 \times 25 = 330,000$ . Figure 34 shows that with DTT included in the medium of the r-protein, not only the fast shoulder in the sedimentation pattern disappeared, but also the  $s_{20,w}$  value decreased from 0.5S to 0.4S. Therefore it seems that the shape of the r-protein may be influenced by the reducing agent. The charge heterogeneity pattern demonstrated by the isoelectric focusing might reflect the heterogeneity pattern of the primary structure. Although

pine seed r-protein was typical in its physicochemical characteristics-- amino acid composition, sedimentation patterns, spectrophotometric properties, and electrophoretic heterogeneties--it showed specific NH<sub>2</sub>-terminal amino acid pattern, having arginine as one of the NH<sub>2</sub>-terminal amino acids.



## VI. SUMMARY AND CONCLUSION

Pine seed ribosomes were reacted with DNS--celite to form DNS-ribosomes, in which 45  $\mu$ moles of dansyl group per mg of ribosomes were bound. In the reaction only the r-protein moiety of the ribosome was dansylated, leaving the RNA intact. The DNS-ribosome had the same sedimentation patterns as the normal ribosomes in the cold, and the temperature-absorbance profiles at 260 nm of the DNS-ribosome and the normal ribosome were similar below 36<sup>o</sup>, indicating that structural integrity of the ribosome was not altered by the dansylation. The dissociation and reassociation patterns of the DNS-ribosome and the normal ribosome were the same at various magnesium concentration levels. Because of this, it has been suggested that the DNS-ribosome may be useful in studying the function(s) of the r-protein in protein biosynthesis.

In the temperature-absorbance profiles, dansylation of the ribosome brought about a lowering of the  $T_m$  by 2.0<sup>o</sup>, and a 0.057 increase in the slope of the differentiated melting curve at the  $T_m$ , indicating that dansylation may decrease the stability of double-stranded helical r-RNA.

When the magnesium concentration was lowered and the ribosome became more unfolded, both the normal ribosome and the DNS ribosome, became more sensitive to ribonuclease. In the case of normal ribosome, the 40S subunit was the most sensitive to ribonuclease, while the 80S was the most resistant to the ribonuclease. As the magnesium concentration was lowered, this difference in ribonuclease sensitivity between ribosomal units decreased. The DNS-ribosome was always more sensitive

to ribonuclease digestion than the normal ribosome. The r-protein may be an important factor for the maintenance of the conformation of the r-RNA and the protection of it from the ribonuclease attack.

More than 20% of the total protein of the crude ribosome could be detached in the washing processes. Accordingly, the ratio of the RNA content to protein content increased as the ribosomes were washed. However, most of the protein was removed in the first washing with buffer B and the second washing with the buffer containing 0.35M  $\text{NH}_4\text{Cl}$ . The structural integrity (judged by sedimentation patterns) was not destroyed by the detachment of proteins. Proteins most readily removed were those whose pI's lay in the range of 6.3-7.5, and they were speculated to be mainly acidic amino acids in amide forms. The most basic (pI, 8-8.5) and the most acidic (pI, 4.5) protein were the least detachable, as shown by the isoelectric focusing patterns of removed proteins. The proteins removed from the ribosomes during each washing process, contained a higher content of acidic amino acids than basic amino acids, whereas the proteins remaining in the ribosome contained a higher content of basic amino acids than acidic amino acid. The major isoelectric focusing bands of the removed proteins (supernatant fractions) overlapped with those of the remaining proteins (ribosomal pellet fractions). The pairs of overlapping proteins, removed and remaining, were presumed to consist of identical protein components. The disc gel electrophoresis patterns (and chromatography patterns by the Kurland group) of the detached proteins were distinguishable from those remaining in the ribosome. From these observations it was speculated that the size and shape of overlapping pairs of proteins are distinguishable from each

other, whereas their pI's are the same. This speculation, with the observation that ribosomal integrity is maintained in the detachment of proteins, supports the theory that r-proteins are composed of multiple copies of fundamental subunit proteins. Ribosomal proteins appear to be composed of a few rather discrete groups. The physicochemical properties of r-proteins in ribosomes might be different depending upon their positions in the ribosome.

Characteristics of pine seed r-proteins were typical, as already discussed in "Results", in their electrophoretic and charge heterogeneity, amino acid composition, sedimentation properties,  $\bar{M}_w$ , and spectrophotometric properties. Amino-terminal amino acids reported have been different according to the source of the r-proteins. As  $\text{NH}_2$ -terminal amino acids, arginine, alanine, and lysine were detected.

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SOME PROPERTIES AND FUNCTION OF  
RIBOSOMAL PROTEINS FROM THE SEEDS  
OF PINUS LAMBERTIANA

Thong-Sung Ko

Abstract

Functional roles of ribosomal proteins (r-proteins) on the structural properties of ribosomes from Pinus lambertiana were studied. For this purpose, r-proteins in the ribosome were dansylated with dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) dispersed on celite (DNS-Cl-celite); sedimentation properties, ribonuclease sensitivity, and the temperature-absorbance profile, of the dansylated ribosomes (DNS-ribosomes) were compared with normal ribosomes; the isoelectric focusing patterns of proteins removed (supernatant fraction) during washing procedures and those of proteins remaining (ribosomal pellet fraction) were investigated; characteristics of extracted r-proteins were examined.

Ribosomal proteins in the ribosome were dansylated, leaving the r-RNA unreacted. Prepared dansyl ribosomes (DNS-ribosomes) had the same sedimentation patterns as the normal ribosomes. The dissociation and reassociation properties of ribosomes were not altered by the dansylation at various magnesium concentrations. In the temperature-absorbance (260 nm) profiles, dansylation of the ribosome brought about a lowering of the  $T_m$  by  $2^\circ$  and a 0.057 increase of the slope of the differentiated melting curve at the  $T_m$ . The DNS-ribosome was more sensitive to ribonuclease digestion than the normal ribosome, under

all conditions tested. From the above observations, it was concluded that: The DNS-ribosome may be useful in the study of the functional role of r-proteins in the protein synthesis processes; the r-protein may be an important factor for the maintenance of the conformation of the r-RNA in the ribosome; ribosomal proteins may protect the r-RNA from ribonuclease digestion. In the case of normal ribosomes, the 40S subunit was the most sensitive to ribonuclease, while the 80S was the most resistant to the ribonuclease. As the magnesium concentration was lowered, this difference in ribonuclease sensitivity between ribosomal units decreased.

More than 20% of the total protein of the crude ribosome could be detached in the washing processes without causing a destruction of the structural integrity of the ribosome as evidenced by sedimentation analysis. Proteins most readily removed were those whose pI's lay in the range of 6.3 - 7.5. The most basic (pI, 8 - 8.5) and the most acidic (pI, 4.5) protein were the least detachable, as shown by the isoelectric focusing patterns of removed proteins. The proteins removed from the ribosomes during each washing process, contained a higher content of acidic amino acids than basic amino acids, whereas the proteins remaining in the ribosome contained a higher content of basic amino acids than acidic amino acids.

The neutral pI's associated with the detached proteins, instead of acidic pI's as would have been expected from the amino acid data, were probably due to the presence of many amide groups, as suggested by the high ammonia peaks from these proteins, and the constant ratio of the content of acidic amino acids to ammonia.

All of the isoelectric focusing bands, from protéins which were removed during washing (removed protein), corresponded to bands which remained with the ribosomal particle. Previous work had shown that the disc electrophoresis patterns of the removed proteins were different from those proteins remaining in the ribosome. Although the primary structure of the removed proteins are the same as those remaining in the ribosome (same pI values), there may be conformational and aggregational differences between these two groups of proteins (different disc gel bands). This speculation, with the observation that the integrity of the ribosomal structure is not destroyed by the detachment of proteins, may lead to the theory that r-proteins are composed of multiple copies of fundamental subunit proteins, and that the protein component patterns in isoelectric focusing might represent the patterns of pI's of these fundamental subunit proteins. The physicochemical properties of r-proteins in ribosomes might be different depending upon their positions in the ribosome.

Characteristics of pine seed r-proteins were typical in their electrophoretic and charge heterogeneity, amino acid composition, sedimentation properties,  $\bar{M}_w$ , spectrophotometric properties. As  $\text{NH}_2$ -terminal amino acids, arginine, alanine, and lysine were detected.