A CHARACTERIZATION OF THE RIBONUCLEIC ACID AND RIBOSOMES OF THE MYXOMYCETE PHYSARUM RIGIDUM

A Thesis

Presented to

the Faculty of the Department of Biology

The University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Microbiology

by

Donald Jungkind

August, 1968

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ABSTRACT

Plasmodium of Physarum rigidum A was grown in axenic shake culture at 24°C in a soluble semi-defined medium. Aliquots were removed at 24 hour intervals and measured for dry weight, protein and RNA content. By day 13, the maximum values were reached. These were 85.0, 51.0 and 8.3 mg, respectively, per 50 ml of media.

Extracts were made in a pH 7.4 buffer containing 0.01 M Mg^{++} ions, and the S°_{20} value of the ribosomes was determined to be 79.6. The magnesium concentration was lowered and the S_{20} values of the subunits were determined to be 37.6 and 57.5 respectively.

The sedimentation values of phenol purified total RNA were obtained using the analytical ultracentrifuge and absorption optics. The boundaries found had S°_{20} values of 5.1, 17.7 and 26.8.

The nucleotide composition of the total RNA and ribosomal RNA was determined on multiple samples by chromatography on AG1-X8 formate resin following alkaline hydrolysis. The total RNA content of guanylic, cytidylic, adenylic, and uridylic acids respectively is 33.67 ± 0.99, 18.82 ± 0.60, 24.09 ± 0.70, and 23.42 ± 0.36. The corresponding composition of ribosomal RNA is 31.62 ± 1.18, 19.33 ± 1.29, 26.25 ± 0.42, and 22.80 ± 0.67.

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Part I

INTRODUCTION

Review of the Literature

The true slime molds, or Myxomycetes, have been observed by scientists for some 150 years (3). Most slime molds are found on decaying organic matter in shady moist areas, although a few species occur in the open (2). They feed on bacteria, protozoa, and other minute organisms.

The sequence of events in the typical life cycle is as follows (3). Under proper conditions of temperature and moisture, the haploid spore germinates, giving rise to one or more myxamoebae. These can undergo numerous cell divisions. At anytime, provided sufficient moisture is present, each can become flagellated to form a swarm cell. The myxamoebae, or swarm cells, fuse to form a diploid zygote which undergoes a series of mitotic nuclear divisions resulting in a multinucleate plasmodium with diploid nuclei. At maturity, the plasmodium thickens and begins the fruiting process. Meiosis occurs and the haploid spores are encased in the stalked fruiting body.

Slime molds in general have been of little direct economic importance. However, they have begun to receive biochemical studies in recent times because they are ideal tools for experimental studies on the mitotic cycle, morpho-

genesis, the chemical changes that govern reproduction, the structure and physiology of protoplasm, and a variety of other fundamental questions (2).

One of the problems that had to be solved prior to intensive biochemical study was how to grow the organisms in axenic culture that was at least semi-defined. Of the 400 or more known species, only about 30 have been induced to complete their entire life cycle in even crude or two membered cultures (1). Pure cultures of Myxomycete plasmodia were usually grown on sterilized ground oats or formalin killed yeast or bacteria (31).

Daniel and Rusch (13) finally succeeded in growing the plasmodium of Physarum polycephalium in pure culture on a chemically defined liquid medium. Henney and Henney developed a semi-defined liquid medium which will grow the plasmodia of several other species of the Physarum genus in pure culture (19). Included in their list of species is Physarum rigidum strain A, which was used for the work described in this thesis.

Earlier chemical studies on the Myxomycetes are reviewed by Martin (31) and Alexopoulos (1). Much of this earlier work dealt with the protoplasmic streaming, pigments, enzymes, and antibiotics. Information about the increase in dry weight and protein during the growth of P. polycephalum on semi-defined or defined media is given by several authors

(8,13). The growth curves show a rapid increase, a leveling off, and then a drop in each of the components studied as a function of time.

The relative amounts of RNA to protein in the plasmodium of P. polycephalum is obtainable from the article of Braun, Mittermayer, and Rusch (6). Their figures give an RNA/protein ratio of about 0.08 in the whole plasmodium.

The work with nucleic acids centered largely around studies of the mitotic cycles in the nuclei of a synchronous plasmodium. The first early work was by Nygaard, Guttes, and Rusch (35) who studied nucleic acid metabolism in synchronous cultures of P. polycephalum. A photographic representation of the morphology and synchrony is given by Guttes, Guttes, and Rusch (18). The polysome patterns and protein synthesis during the mitotic cycle of P. polycephalum is given by Mittermayer, Braun, Chayka, and Rusch (32). They used sucrose density gradients and 70 S bacterial ribosomes as markers in a swinging bucket centrifuge to determine that the P. polycephalum ribosomes must be of the 80 S class. However, in the study of the ribosomal subunits, they reported values of about 30 and 50 S instead of the usual 40 and 60 S values for 80 S ribosomes. The quantity of subunits was increased by decreasing the magnesium ion concentration. Many aggregated ribosomes in the 10-20 unit range were found. Polyribosomes were most often hexamers or

less in size. The RNA/protein ratio of the ribosomes was given as 0.92. Braun, Mittermayer and Rusch (6) reported an RNA/protein ratio of 0.25, but this was attributed to contaminating mitochondria and nuclei.

A report by Ashworth from M. Sussman's lab (4) stated that the ribosomes of the cellular slime mold Dicteostelium discoideum were of the 70 S class, like the bacterial ribosomes. An earlier report from the cellular slime mold Polyspondylium pallidum set the value at 80 S (40).

Using P. polycephalum, Cummins, Weisfield, and Rusch (12) determined the base composition of the microsomal RNA. Rusch's group also found a DNA-dependent RNA polymerase in the isolated nuclei (33). Using the intact DNA, four nucleoside triphosphates, magnesium ions and polymerase, they synthesized RNA in vitro that did not exceed 5 S RNA in size. The base composition of this was slightly different from the ribosomal RNA.

There were no reports in the literature that the purified RNA of any Physarum species has been studied in the analytical ultracentrifuge. Using sucrose gradients and Escherichia coli RNA as a marker, Mittermayer, Braun, and Rusch (34) concluded that the ribosomal RNA of P. polycephalum has S values of about 17 and 27. The RNA exhibited the usual sensitivity to RNAse. As expected P. polycephalum RNA synthesis was sensitive to exposure to Actinomycin D.

Later (7), the same group gave a more extensive report on the RNA purification. Again they used sucrose density gradient centifugations to determine the S values. They found that RNA extraction by the cold phenol method gave satisfactory results with no DNA contamination. However, they frequently had trouble with RNA degradation. Sodium dodecyl sulfate was not necessary to the extraction. Sixty-eight percent of the RNA was removed by the first phenol extraction. The RNA was found to be 13% soluble RNA and 87% ribosomal RNA. This ribosomal RNA was 31% 17 S and 69% 27 S. They detected heavier RNA up to 50 S, but the predominant values were 33 S and 19 S. They thought this could be the precursor of ribosomal RNA.

In summary, since the ability to grow P. rigidum is such a recent development, no literature has accumulated about its RNA and ribosomes. Nucleic acid studies have been made on the related organism P. polycephalum. The S values of the RNA and ribosomes were determined using sucrose gradients and were not studied in the analytical ultracentrifuge. These studies have stood almost alone as representative of the entire class Myxomycetes and there is an occasional small conflict in information. The S values of the ribosomes in the cellular slime molds has been reported as both 80 and 70 S. In the acellular slime mold, P. polycephalum, the expected 80 S value was reported, but the

values for the subunits fit those of a 70 S ribosome. There seems to be a need to clarify and reinforce the data already obtained on the Myxomycetes.

Statement of Problem

The Myxomycetes have been a little studied group of organisms until relatively recently due to an inability to grow them in pure axenic culture. The growth characteristics of the Myxomycete Physarum rigidum strain A were determined with respect to RNA on the semi-defined soluble medium developed by Henney and Henney (19).

An analytical characterization of the RNA and ribosomes of the organism was the second part of the thesis problem. This research included a base composition study of the total and ribosomal RNA.

The RNA was extracted and purified, and sedimentation values determined by absorption optics in the Spinco Model E analytical ultracentrifuge. The chemical composition of the RNA was studied and the extinction coefficients at 260 mμ were established.

The sedimentation values for the ribosomes and the subunits were determined using Schlieren optics in the analytical ultracentrifuge. The chemical composition of the ribosomes were also studied.

Part II

MATERIALS AND METHODS

Chemicals

All chemicals were reagent grade in purity unless otherwise specified. All were obtained from the J. T. Baker Chemical Co., Phillipsburg, N. J. unless noted otherwise.

Equipment

All optical density (O.D.) measurements were made in the Beckman DU-2 Spectrophotometer (Beckman Instruments Co., Fullerton, Calif.) using matched 1X1 cm standard silica cuvettes (Beckman Instrument Co.).

Centrifugations were made in the Servall (Ivan Servall Incorporated, Norwalk, Conn.) Model RC-2 centrifuge set at $0^{\circ}C$ unless otherwise stated.

Measurements of pH were made on a Beckman Zeromatic II pH meter.

Water

All water used in the procedures described was double distilled using a Corning Model AG-3 (Corning Glass Co., Corning, N. Y.) glass still.

Protein Determination

The protein determination was by the method of Lowry *et al* (29). Bovine serum albumin (Armour Pharmaceutical Co.,

Kankakee, Ill.) was used as a standard by weighing out 0.050 gm and gently dissolving in 20 ml of 0.02 N HCl (Matheson Coleman and Bell, East Rutherford, N. J.). The absorbance at 278 mμ on a 1:10 dilution was determined. The amount of protein in the solution was calculated using the following relationship (23):

Absorbance = 0.64 liter $\cdot$ g^{-1} $\cdot$ cm^{-1} at 278mμ

The standard was made 0.4 N in NaOH (Matheson, Coleman and Bell) using a 4.0 N solution. Prior to testing, all unknown samples were dissolved in 0.4 N NaOH by heating to 100°C for 10 minutes. Four-tenths N NaOH was used to make all the dilutions and for a blank.

The reagents used in the test are made as follows: alkaline tartrate solution (reagent A) is made by adding 20 gm of sodium carbonate, 0.5 gm of sodium or potassium tartrate (Fisher Scientific Co., Fairlawn, N. J.), and 4.0 gm of sodium hydroxide with the volume brought up to 1000 ml with glass distilled water.

Reagent B is a solution of 1 gm of $CuSO_4 \cdot 5\ H_2O$ in 1000 ml of water.

Reagent C is Folin - Ciocalteu phenol reagent (Anderson Laboratories, Ft. Worth, Tex.) diluted with an equal volume of water.

Each day, one part of B plus nine parts of A were mixed to form reagent D.

The test was performed by adding 0.5 ml of a standard, sample, or blank to a test tube. Five ml of alkaline copper reagent D was then added. This was mixed and allowed to stand at room temperature for 10 minutes. Last, 0.5 ml of the diluted phenol reagent C was added and mixed immediately. After 30 minutes, the O.D. of the tubes were read at 700 mμ. The standard curve was plotted and the unknown samples were compared to it.

D (-) Ribose Determination

The orcinol test for D (-) ribose (10,14) was modified and used as explained below. The standard used was a solution of D (-) ribose (Sigma Chemical Co., St. Louis, Mo.). This was kept at $4^{\circ}C$ and diluted with water to give a series of concentrations from 50 to 1.25 μg per ml.

The orcinol acid reagent was made by adding 2 ml of a 10 percent $FeCl_3 \cdot 6\ H_2O$ solution to 400 ml of concentrated HCl.

The orcinol reagent was a 6 percent solution of purified orcinol (Sigma Chemical Co.) in 95 percent ethanol.

The test was run by adding 3 ml of standard, sample, or water blank to a test tube. Six milliliters of the orcinol acid reagent was added and mixed. Four-tenths milliliter of the 6 percent alcoholic orcinol was added next, and the tubes were heated in a boiling water bath for 20 minutes. The liquid level in the tubes had to be submerged below the

level of the boiling water for consistent results. The O.D. readings were made at 660 mμ. The standard curve was established and the unknown samples were compared to it.

Inorganic Phosphorous Determination

The inorganic phosphorous content was determined by the method of Fisk and SubbaRow (16) as modified by the Sigma Technical Bulletin number 670 (Sigma Chemical Co.). The total phosphorous in the samples was converted to inorganic phosphorous by the method described by Leloir and Cardini (25).

For the sample preparation, one or two ml of 5.0 N H_2SO_4 was added to a known volume of sample. The mixture was evaporated in a test tube over a free flame. Care was taken to avoid splattering. When the contents nearly reached dryness, 0.5 ml of a 2.0 N HNO_3 solution was added and heated until the white fumes disappeared and a colorless residue remained. All traces of the nitric acid were evaporated out of the test tube. Two ml or more of water was added and the tube placed in a boiling water bath for 5 minutes with occasional shaking to rinse the sides of the tube. The phosphorous content was determined on this or on an appropriate dilution.

A phosphorous standard curve solution was made by adding KH_2PO_4 to a concentration of 40 μg per ml in water. This was used full strength and in dilutions down to 2 μg/ml.

The test reagents were made as follows:

An acid molybdate solution was made by making a 1.25 percent $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ solution in 2.5 N H_2SO_4.

A Fisk and SubbaRow reducer solution was made by dissolving 1.0 gm of solid reducer (Sigma Chemical Co.) in 6.3 ml of water.

For the actual test, 2.0 ml of sample, standard, or water blank was added to a test tube. To this was added 3.0 ml of water and 1.0 ml of acid molybdate solution. The contents were mixed by shaking and 0.25 ml of the reducer solution was added. The contents were mixed by inversion and allowed to stand at room temperature for 10 minutes. The O.D. at 660 mμ was then read immediately.

Nuclease Inhibitors

A bentonite no. 325 (American Colloid Co., Skokie, Ill.) suspension was prepared by a modification of Petermann's procedure (38). A suitable amount was weighed and added to a known volume of buffer which was to be used. This was equilibrated at 4°C overnight and centrifuged 5 minutes at 3000 X g and the pellet discarded. The supernatant was re-centrifuged at 8000 X g and the supernatant discarded. The pellet was resuspended in a known volume of buffer and the approximate mg/ml concentration estimated. This was stored at 4°C.

Macloid (Baroid Div. National Lead Co., Houston, Tex.)

was prepared as described by Stanley and Bock (44). One hundred milliliters of boiling water was placed in a pre-warmed blender. Five grams of the purified hectorite was added and the mixture blended until a thick colloid formed. The mg/ml concentration was calculated and colloid stored at 0-4°C.

Polyvinyl sulfate (K and K Laboratories, Inc., Plane-view, N. Y.) was also occasionally used at a concentration of 20 to 50 µg/ml (21), but bentonite and Macloids were relied on as the main nuclease inhibitors.

Source of the Organism

The organism selected for the studies was *Physarum rigidum* strain A. The fruiting bodies were collected and the plasmodia developed and grown as described by Henney and Henney (22).

Media and Growth of the Organism

The media of Henney and Henney (19) was used to grow the plasmodium in liquid and on solid semi-defined media. A study was run to determine when would be the best time to harvest the cultures. The growth curve is established by measuring the increase in dry weight, ribonucleic acid (RNA), and protein. Eighty milliliters of complete media (19) was placed in each 500 ml Erlenmeyer flask. *P. rigidum* A, which was well established in liquid culture, was inoculated

into each flask. The inoculum size was 30 μg of protein per 80 ml of media. Samples were withdrawn on days 3-9, 11, 13, and 15, with a variation of ± 1.5 hours from the original hour of inoculation. The samples were centrifuged at 8,000 X g for 10 minutes. The supernatant was poured off and the pellet treated by the method of Ogur and Rosen as described by Leslie (26), modified and performed as follows: the pellet was crushed and extracted three times with 0.5 N perchloric acid (PCA) for 20 minutes at 0°C. The light yellow supernatant was discarded after centrifugation at 15,000 X g. The pellet was crushed and suspended in 0.5 N PCA for 50 minutes at 70°C. This was centrifuged and the supernatant saved. The process was repeated on the pellet, and the supernatants pooled for RNA determinations by the orcinol method. The calculations were made with the following formula:

$$\frac{V \cdot D \cdot R \cdot 50}{W \cdot P \cdot C \cdot 1000} = \text{Mg RNA/50 ml media}$$

V = Volume of combined 70°C PCA supernatant.

D = Subsequent dilutions of the supernatant.

R = μg D (-) ribose/ml as determined from the orcinol curve.

W = Number of mls withdrawn from the sample flask.

P = Percentage of purines in total RNA as established by the base ratio studies (57.76 percent).

C = Percent D (-) ribose in the total RNA established from the base ratio studies (43.81 percent).

50 = Multiplication factor to correct for 50 ml media.

1000 = Correction factor to mg.

The protein pellet from above was saved for protein measurements as previously described.

Dry weights were determined on separate 5 or 10 ml aliquots withdrawn at the same time as the RNA and protein sample. After harvesting by centrifugation, the pellets were washed with distilled water into preweighed aluminum weighing pans. These were dried for 18 hours at 110°C in an oven (Precision Scientific Co., Model 17, Chicago, Ill.). Splattering did not occur at this temperature. The weight was determined to the fourth decimal place on a Mettler type H 15 single pan balance (Mettler Instrument Corp., Hightstown, N. J.).

The percentage of water was determined near the peak of the curve by also weighing the fresh plasmodia after draining off excess moisture with filter paper.

The measurements of RNA, protein, and dry weight were related back to the total content per 50 ml of medium. At the peak of the curve, the RNA and protein as a percent of the dry weight were calculated.

With the growth characteristics determined, the characterization of the RNA and ribosomes was begun.

Ribosome Characterization

For the ribosome work, cultures of P. rigidum A were inoculated such that the peak of growth occurred at about 7 days. At this point, the cultures were harvested by centrifugation at 8,000 X g. If too much extracellular slime adhered to the pellets, it was rinsed away with fresh liquid media (19), modified by omitting the yeast extract, peptone, and hematin. All further preparations and procedures were made at 0 - 4°C unless otherwise noted.

The first analysis considered is the sedimentation values of the ribosomes and the subunits. These were determined from the direct extracts by the use of the Spinco Model E analytical ultracentrifuge and Schlieren optics (Spinco Div., Beckman Instruments, Palo Alto, Calif.). For this work, the plasmodia were ground in a 16 ml ground glass homogenizer (Belco Biological Glassware, Vineland, N. J.) with 1/10 W/V of alumina (Sigma Chemical Co.) and one of the buffers listed below. The total volume was kept to a minimum during the extraction procedure in order to keep the ribosome concentration as high as possible. The buffers used are as follows:

A. 0.01 M potassium phosphate (Mallinckrodt Chemical Works, St. Louis, Mo.), pH 7.4, plus 0.01 M magnesium chloride.

B. 0.01 M Tris - HCl (Sigma Chemical Co.), pH 7.4,

plus 0.05 M potassium chloride, 0.005 M magnesium acetate, and 2 μg/ml of ribonuclease A (Sigma Chemical Co.).

C. 0.01 M Tris - HCl, pH 7.4, 0.05 M potassium chloride and 0.05 M magnesium acetate.

D. 0.01 M Tris - HCl, pH 7.4, 0.25 M potassium chloride.

E. 0.01 M Tris - HCl, pH 7.4, 0.25 M potassium chloride, and 0.01 M magnesium acetate.

Occasionally Brij 58 (Atlas Chemical Industries, Inc., Wilmington, Del.) was added to a final concentration of 0.2 percent (15), but did not seem necessary to get large quantities of membrane free ribosomes.

The extracts were cleared by two centrifugations at 20,000 X g for 10 minutes. The sedimentation values on the ribosomes in the supernatant were determined as follows. About 0.8 ml of the extract was added to a Kel-F cell (Spinco Div., Beckman Instruments) of the analytical ultracentrifuge. The standard AN-D rotor was used at a speed of 42,040 RPM and a temperature of 20°C. Photographs were made every 2 minutes on Metallographic glass plates (Eastman Kodak Co., Rochester, N.Y.) with the Schlieren optical system and an exposure of 3 seconds. These plates were developed for 7 minutes in Kodak D-19 High Contrast Developer and fixed normally.

The calculation of the S^{o}_{20} values is as described by Schachman (41) and Svedberg and Pedersen (45).

Using the Nikon Shadowgraph Model C (Nikon Inc., Nipponkogaku K.K., Japan), the distance in centimeters from the reference band to the peak or boundary was measured. This was then divided by the magnification factor due to the optics of the centrifuge. This was 2.1656 for the horizontal axis and 3.5369 for the vertical axis. To this distance was added the distance from the center of the rotor to the reference band itself. This was 5.70 cm, and an additional 0.01 cm was added to compensate for the expansion of the rotor during centrifugation. The data was fitted into the following formula (41):

$$S_{20} = \frac{2.303\ (\text{Log}\ X_n - \text{Log}\ X_o)}{W^2\ (t_2 - t_1)}$$

X = distance in cm from axis of rotation

t = time in seconds

$S = 10^{-13}$ cm/sec/unit field

$W^2 = (2\ \pi \cdot \text{RPM}/60)^2$

At a speed of 42,040 RPM, the above formulas can be simplified such that $S_{20} = 1.976 \times 10^{-9} \times$ slope. The S_{20} values were corrected for ribosome concentration by making dilutions of a concentrated direct extract and plotting the S_{20} values obtained versus the dilution. The concentrations

used were 1X, 2/3X, 1/2X, 1/3X. The S values at each point were corrected by the method of least squares and the line connecting the points extended to give the S^{o}_{20} value. The subunits and dimers were not present in sufficient quantities to permit a direct determination of the S^{o} value. For a rough estimation of the S^{o}_{20} value of the subunits and other peaks, the percent change in value between the S_{20} and the S^{o}_{20} of the single ribosome peak at a certain concentration was applied to the S_{20} values of the subunit and dimer peaks with similar concentrations.

Chemical Analysis of Ribosomes

For chemical analysis, extracts were made in the cold as before, using the following buffer system: 0.001 M Tris-HCl pH 8.0, 0.25 M sucrose, 0.25 M potassium chloride, 0.001 M magnesium acetate, 0.3 % Brij 58, and 0.2% bentonite.

The extracts were cleared by two centrifugations for 10 minutes at 20,000 X g. The ribosomes were then pelleted by centrifugation at 88,500 X g for 2.75 hours in the 30 K head of the Spinco Model L Preparative Ultracentrifuge (Spinco Div., Beckman Instrument Co.). The ribosomes were resuspended with a precooled 16 ml glass homogenizer (Belco Biological Glassware) in the following buffer: 0.02 M potassium bicarbonate, and 0.001 M magnesium acetate (39). This was adjusted to pH 8.0 if necessary with a few drops

of 0.3 N potassium hydroxide.

The ribosomes were pelleted as before, resuspended, and washed up to two more times using a 0.01 M Tris - HCl, pH 7.4 buffer made 0.25 M in potassium chloride and 0.01 M in magnesium acetate.

Chemical Analysis

The ribosome suspension was well mixed and aliquots withdrawn for chemical analysis to determine the RNA to protein ratio of the sample. Protein determinations were made in triplicate, RNA was determined in duplicate by the orcinol test, and phosphorous was determined in duplicate by the methods previously described. All calculations were related back to the amount of each per ml of ribosome suspension. The following procedure is a method used for estimating the amount of RNA present by the optical density at 260 mμ. To 3.5 ml of ribosomes was added 0.5 ml of 3.0 N KOH. This was hydrolyzed for 18 hours at 37°C. The protein was precipitated with 0.5 ml of 5.0 N PCA. The precipitate was then centrifuged and the supernatant saved. The pellet was washed with 2.5 ml of 0.5 N PCA. The supernatants were combined and the O.D. 260 mμ of a 1:100 dilution was measured. This was compared to an extinction coefficient established using purified P. rigidum RNA hydrolyzed in the same manner. This is described later in this section.

The RNA content per ml of ribosome suspension was calculated using the three different methods - RNA hydrolysis, D (-) ribose, and the phosphorous determination. This was then compared to the protein value and the relative percentages established.

RNA Base Ratios

The RNA base ratios were determined on total and ribosomal RNA. The bases were separated by the method described by Cohn (11). The sample preparation procedure was that of Smith (42). The extinction coefficients of the nucleotides at an acidic pH were those of Beaven, Holiday and Johnson (5). The procedures used are summarized below.

For the total RNA sample preparation, 7 day old plasmodia were harvested by centrifugation as described before. All sample preparation steps were done at 0 - 4°C. The organism was ground with alumina and buffer in a mortar and pestle until a well extracted thick slurry was formed. The buffers used were 0.01 M potassium phosphate pH 7.4, with 0.01 M magnesium chloride, or 0.01 M Tris - HCl, pH 7.4, with 0.05 M potassium chloride and 0.01 M magnesium acetate added. The extract was cleared by centrifugation at 15,000 X g for 10 minutes. The supernatant was brought to 0.5 N with 5.0 N PCA. This was left in the cold for 15 minutes and then centrifuged as before. The supernatant was dis-

carded and the pellet was crushed and washed three more times in cold 0.5 N PCA. The final pellet was crushed in 10 - 15 ml of 0.3 N KOH and hydrolyzed at 37°C for 18 hours. The mixture was then brought to 0.5 N in PCA and the unhydrolyzed materials precipitated by centrifugation. The supernatant containing the nucleotides of the hydrolyzed RNA was brought to pH 9 - 10 with 0.3 N potassium hydroxide and cooled to 0 - 4°C. The precipitated potassium perchlorate was centrifuged out and the supernatant saved for quantitation of the RNA by the O.D. 260 mμ method described later. The sample was frozen at minus 20°C until needed.

The ribosomal RNA sample preparation involved pelleting and washing the ribosomes as previously described. The ribosomes were finally suspended in 0.3 N KOH for hydrolysis and subsequently handled like the total RNA sample.

For the preparation of the column, Bio-Rad AG1 - X8 formate resin, 200 - 400 mesh (Bio - Rad Laboratories, Richmond, Calif.) was suspended in distilled water and allowed to settle nearly to completion. The fines still in the supernatant were poured off and the process repeated several times. The resin was then resuspended in 90 percent formic acid (Matheson, Coleman, and Bell) and allowed to settle. The resin was washed with successive rinses of distilled water until all traces of acid were gone. The prepared resin was stored at 4°C under water until ready

for use. Any resin unused after two weeks was discarded. Just prior to use, the resin was degassed by warming, plus three rinses in 22°C distilled water which had been subjected to a vacuum with a water aspirator apparatus.

The column used had a sintered glass bottom and an inside diameter of 1 cm. This was clamped and adjusted with a plumb bob until it had no tilt. The column was then filled with degassed water and the resin poured in to a height of 3.5 cm ± 0.3 cm. The water was drained until it was about 2 cm above the upper surface of the resin.

The elution buffers were prepared as follows:

A. Formic acid, 0.005 M, was used to elute any nucleosides and other ultraviolet absorbing contaminants.

B. Formic acid, 0.025 M, was used to elute cytidylate.

C. Formic acid, 0.10 M, was used to elute adenylate.

D. Formic acid, 0.01 M, made 0.05 M with respect to ammonium formate (Matheson, Coleman, and Bell) was used to elute uridylate.

E. Formic acid, 0.10 M, made 0.2 M with respect to ammonium formate, used to elute guanylate.

All buffers were warmed to room temperature and degassed prior to use.

To begin the nucleotide determination, a suitable sample volume, calculated to contain a total O.D. 260 mμ of 80-120, was placed on the column without disturbing the surface of

the resin. Also the resin bed was never allowed to run dry. Most of the sample was allowed to enter the resin bed before adding 5-10 ml of water. This was followed by 15 ml more of water, and then by the buffers required to elute in the following order: contaminating nucleosides, cytidylate (C), adenylate (A), uridylate (U), and guanylate (G).

A 3.19 ml fraction was collected in 46 drops with an LKB automatic fraction collector, Type 7,000 (LKB Produkter AB, Stockholm, Sweden). Each tube was read for the O.D. 260 mμ. The O.D. 280 mμ was also measured at intervals and the O. D. 280/260 mμ absorption changes were used to confirm the nucleotide identies of the peaks.

The commercially prepared nucleotides, cytidylic acid, adenylic acid, uridylic acid, and guanylic acid, (Sigma Chemical Co.) were put in solution and the O.D. 260 mμ of each was measured. A known volume of each was added to a column and the percent recovery was calculated as follows:

$$\frac{\text{total recovered O.D.}}{\text{total loaded O.D.}} \times 100 = \% \text{ recovery}$$

The base ratio of Escherichia coli B total RNA was determined. The cells were grown in the defined media of Spahr and Tissieres (43). The base ratio was compared to previous values found for E. coli B total RNA (37).

To calculate the base ratio of any sample, the O.D. 260

mμ of each fraction in the series was plotted against the respective fraction number on a graph. With the elution peaks thus visualized, the O.D. 280/260 mμ ratios were calculated and inserted at the appropriate points. The graphs were then dissected into the four major nucleotide peaks and all optical densities belonging to each peak were summed. Each of these sums was then divided by its corresponding extinction coefficient as mentioned earlier. The moles of each nucleotide were added for a total, and each nucleotide was calculated as a percentage of this whole. Other calculations made were the G + C sum, the purine/pyrimidine ratio, the A + U/G + C ratio and the 6-amino-nucleotide/6 keto-nucleotide ratio.

The average molecular weight of all the nucleotides in each type RNA was calculated by multiplying each base percentage by its respective molecular weight and summing the results. From this was calculated the percentages of D (-) ribose and phosphorous expected in the RNA.

Extraction and Purification of RNA

Cultures of P. rigidum A were grown until a heavy growth was obtained without entering the terminal slime stage. This was at about 7-8 days. The plasmodia was centrifuged at 1,800 X g for 10 minutes at room temperature and the supernatant discarded. The plasmodia was rinsed in the modified

media described in the ribosomal section, and was then placed in a glass homogenizer. This was plunged into an acetone-dry ice bath until ready to use. All further extraction procedures described below were performed at 0°-4°C. All glassware and chemicals were precooled.

Glassware was cleaned by washing with soap and water. It was then soaked in acid dichromate cleaning solution for one to two hours and rinsed with tap water. The glassware was then soaked briefly in a 0.1% sodium dodecylsulfate (SDS) (Nutritional Biochemicals Corp., Cleveland, Ohio), 0.1% Disodium ethylenediaminetetraacetate (EDTA) solution, and rinsed in hot tap water followed by a double rinse in distilled water. The glassware was then covered with aluminum foil and baked at 200°C for two hours. Finally, it was stored at 4°C until ready for use.

All plastic items were treated as glasswear except that they were sterilized in the autoclave.

The Model E ultracentrifuge Kel-F cell and quartz windows were soaked in a warm 0.1% SDS - 0.1% EDTA solution, rinsed with distilled water, and dried. Finally they were rinsed in acetone-alcohol mixed 1:1 followed by distilled water. They were then vacuum dried.

The dialysis tubing was prepared by boiling 5 minutes in the 0.1% SDS - 0.1% EDTA solution. It was then rinsed and boiled in two changes of distilled water for a total of

15 additional minutes.

Presterilized plastic gloves were washed to remove talcum powder and were worn throughout all stages of the RNA preparation, including the cleaning of glassware, and making of solutions that were to be used in the RNA extraction.

As a summary of the precautions necessary for the isolation of high molecular weight RNA, all precautions were taken to make sure that everything which came into contact with the RNA was clean, cold, and free of nucleases. The work was organized such that the entire extraction process was 6-8 hours.

The extraction process itself is a procedure modified from Kirby's original cold phenol procedure (24). The plasmodia was partially thawed and an equal volume of 0.01 M Tris - HCl pH 7.4, containing 0.005 M magnesium chloride, and 0.05 M potassium chloride, was added. Bentonite, Macloids, and SDS were added to a final concentration of 0.2% W/V for each. The centerpiece of the 65 ml glass tissue homogenizer (Kontes Glass Co., Vineland, N. J.) was turned at about 800 RPM for 20 up and down strokes. Quickly an equal volume of buffer equilibrated phenol (Matheson, Coleman, and Bell) was added. Both redistilled and non-redistilled phenol were used on two extractions. The mixture was vigorously shaken for two minutes and then transferred to a capped Nalgene centrifuge tube (Ivan, Sovall Inc., Norwak, Conn.). The tube

was shaken an additional 25 minutes and centrifuged 10 minutes at 2000 X g. Both the upper aqueous layer and the lower phenol layer were saved. A half volume of the extraction buffer was added to the phenol layer and shaken for 15 minutes to re-extract for RNA. This was centrifuged as before and the supernatant from this added to the first. Bentonite and Macloids were re-added to give a concentration of 0.1 percent W/V for each. The phenol extraction procedure was repeated until no protein interface remained. This required at least four additional extractions. About 5 - 10 percent of the aqueous layer was left behind each time in order not to carry over any protein.

The RNA was precipitated with two volumes of absolute ethanol made 2 percent W/V in potassium acetate and pre-cooled to minus 20°C. The mixture was placed in the freezer for 1.5 - 2 hours. The precipitated RNA was pelleted by centrifugation at minus 20°C for 10 minutes at 5000 X g. The RNA pellet was washed once with 10 - 15 ml of frozen 70 percent ethanol made 2 percent in potassium acetate. This was centrifuged as before and the RNA pellet redissolved in one of the following buffers: 0.017 molal potassium phosphate pH 7.0 plus 0.01 M EDTA, or 0.01 M sodium acetate pH 4.6 plus 0.1 M NaCl (38).

The RNA was reprecipitated with the absolute ethanol potassium acetate solution and centrifuged as described

previously. The RNA pellet was redissolved in a minimum quantity of the desired buffer. The sample was placed securely in the specially prepared dialysis tubing and dialyzed three hours against 200 volumes of the same buffer. This dialysis procedure was repeated two more times. The RNA sample was then quantitated by its O.D. 260 mμ using the relationship discussed later. Macloids (0.2% W/V) or polyvinyl sulfate (20 μg/ml) were occasionally added to a portion of the sample. The sample was then used immediately or frozen at minus 20°C.

To change the buffer which the RNA was dissolved in, the RNA was reprecipitated as described above, dissolved in the desired buffer and then dialyzed against the same.

Analytical Ultracentrifuge Study of Purified RNA

Absorption optics were used, and the sedimentation values were determined at 20°C. The centrifugations were made in the standard AN - D rotor at a speed of 42,040 RPM. Concentrations of RNA ranged from 150 to 37 μg/ml. The length of exposure was from 30 to 10 seconds every four minutes. The time of the run varied from 28 to 80 minutes with an average of about 48. Photographs were made on Commercial Safety Film (Kodak) and developed in Kodak D-19 for 7 minutes.

The optical density changes of the photographs were

traced in a Beckman Model RB Analytrol equipped with a film densitometer attachment (Beckman Instruments, Inc.). The S values of the boundaries were calculated as described in the ribosome section of this thesis. Modifications were as follows. The mid-point of the analytrol tracing slopes were used to determine the distance of the boundary from the reference lines. The lower concentrations were assumed to give the S° values. The S°_{20} values were averaged for each boundary in each buffer type.

To check whether the absorption boundaries obtained were actually due to DNA, a nuclease inhibitor free sample, dissolved in the acetate buffer, was prepared. Ribonuclease free deoxyribonuclease (Worthington Biochem. Uorp., Freehold, N. J.) was added to a concentration of 240 µg/ml and magnesium chloride was added to 0.01 M. This was incubated 1.5 hours at 37°C. The sample was then analyzed in the analytical ultracentrifuge to check for a decrease in the sedimentation values. If any occurred, or if the boundaries completely disappeared, then the boundaries under study were actually due to DNA.

Chemical and O.D. Characterization of RNA

An RNA sample that had never been in the phosphate buffer was studied for other properties. The orcinol test previ-

ously described was run on the RNA. The amount of D (-) ribose per ml of RNA solution was calculated from this using the expected percent of D (-) ribose in the RNA as determined previously from the base ratio. The calculations were set up as follows:

$$\frac{\mu g \text{ D (-) ribose/ml} \times 100}{\text{percent D (-) ribose in total RNA}} = \mu g \text{ RNA/ml}$$

To check whether all of the D (-) ribose had been measured in a direct analysis of the RNA by the orcinol test, the experiment below was performed.

An RNA sample was made 0.3 N in KOH and hydrolyzed for 18 hours at 37°C. The solution was brought to 0.5 N with respect to PCA. No protein precipitate formed, but the sample was centrifuged at 15,000 X g for 10 minutes. An orcinol test was performed on the supernatant and related to the amount of D (-) ribose per ml of RNA suspension. A higher value in the prehydrolyzed sample would indicate that not all the RNA was hydrolyzed under the conditions of the orcinol test.

An appropriate dilution of the hydrolyzed RNA was made and the O.D. measured at 260 mμ. The total O.D. 260/ml, and the mg/ml of RNA, as determined by the orcinol test, were used to calculate an extinction coefficient of the hydrolyzed RNA. This was used in calculations of the RNA content of the ribosomes as described previously.

The unhydrolyzed RNA was diluted 1:100 into a buffer of 0.01 M Tris-HCl, pH 7.4, 0.01 M magnesium acetate, and 0.05 M potassium chloride. The absorption profile from 202 to 300 mμ was measured using the same buffer as a blank. The O.D. at 260 mμ was rechecked, and an extinction coefficient established as with the hydrolyzed RNA.

The amount of phosphorous per ml of the RNA suspension in the acetate buffer was determined as described earlier. The expected percent phosphorous in the RNA, as calculated from the base ratio data, was compared to the experimentally determined percentage using the phosphorous and the orcinol test data. This served as a cross check on both tests.

Part III

RESULTS

Protein, D (-) ribose, and Phosphorous Standards

The protein, D (-) ribose, and the phosphorous standard curves are shown in Figures 1, 2, and 3 respectively.

Growth of the Organism

P. rigidum A growing on solid and liquid media is shown in Figures 4 and 5. In Figure 5, the microplasmodia growing in liquid were removed to a 50 mm Petri dish for photography.

With respect to the growth curve, the orcinol test had a slightly brown tint when compared to the emerald green colors of the standards. This had been reported previously in *P. polycephalum* (34). Various methods were tried to prevent this brown tint. The use of $CuCl_2$ as described by Ceriotti (9) instead of $FeCl_3$ had no effect. Extraction of the green color by shaking with N-amyl alcohol (9) also carried along this color. The protein and RNA growth curves were similar and thus the tint did not hinder picking the proper harvest point for further studies.

The results of the growth study are shown in Table 1. These results are also plotted as a growth curve in Figure 6. Day 13 was used as most representative of the cultures at the peak of growth, and the chemical analysis results are shown in Table 2.

Fig. 1. Protein standard curve using bovine serum albumin and the Folin-phenol method.

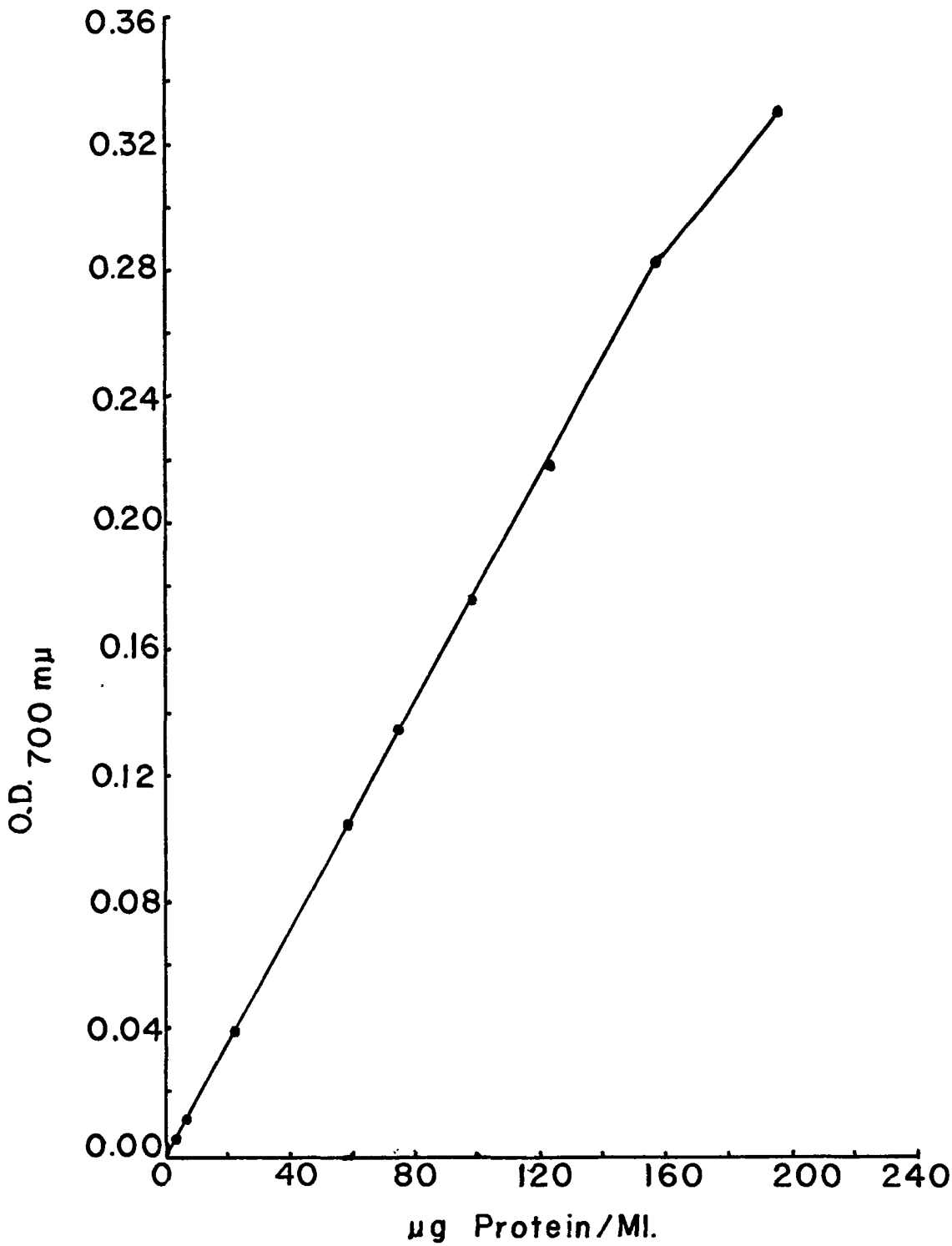

0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
O.D. 700 mμ
0
40
80
120
160
200
240
μg Protein/Ml.

Fig. 2. D (-) ribose standard curve when measured by the orcinol reaction.

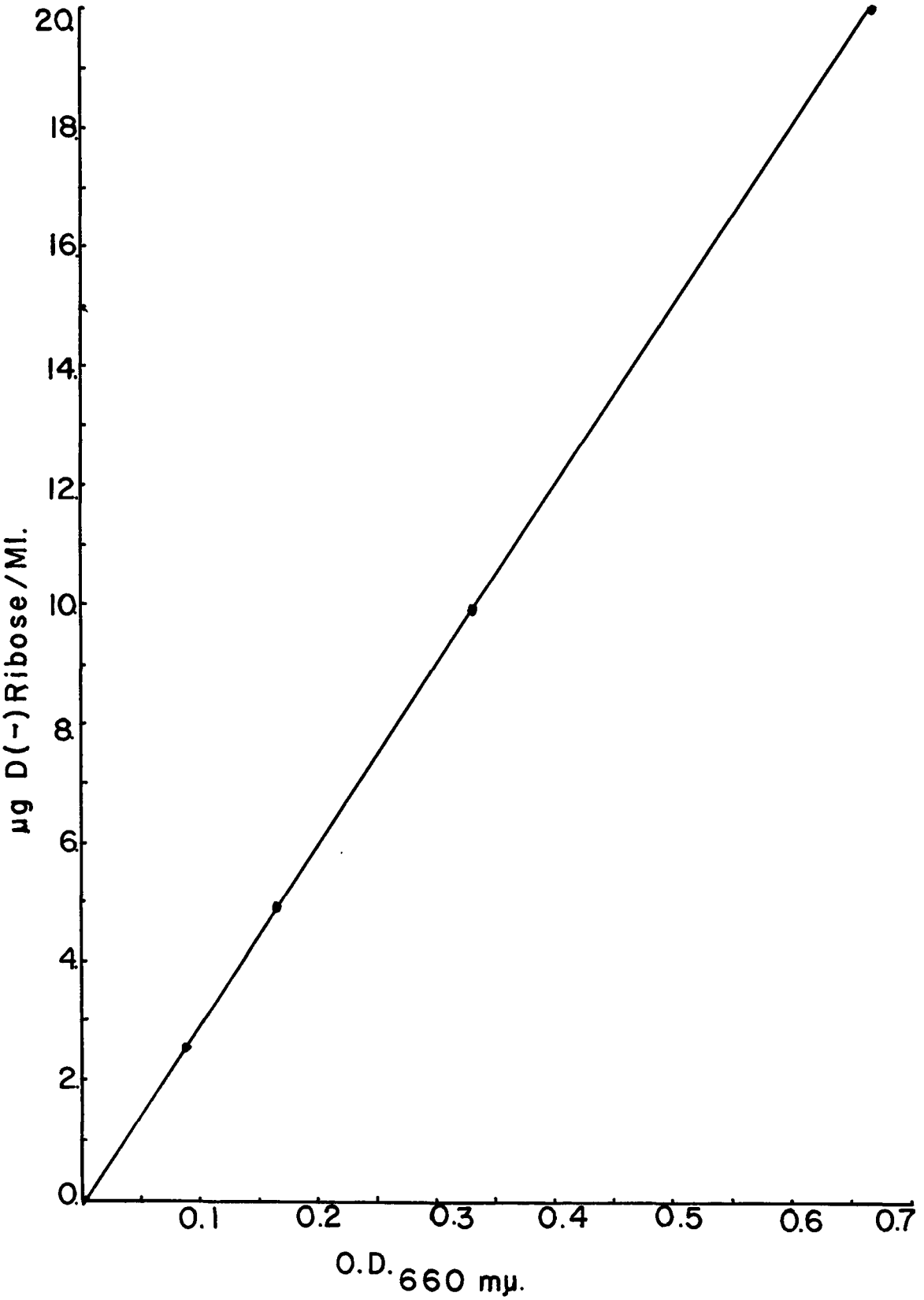

20
18
16
14
12
10
8
6
4
2
0
µg D(-) Ribose/Ml.
0.1
0.2
0.3
0.4
0.5
0.6
0.7
O.D. 660 mµ.

Fig. 3. Inorganic phosphorous standard curve measured by a modification of the Fisk and SubbaRow procedure.

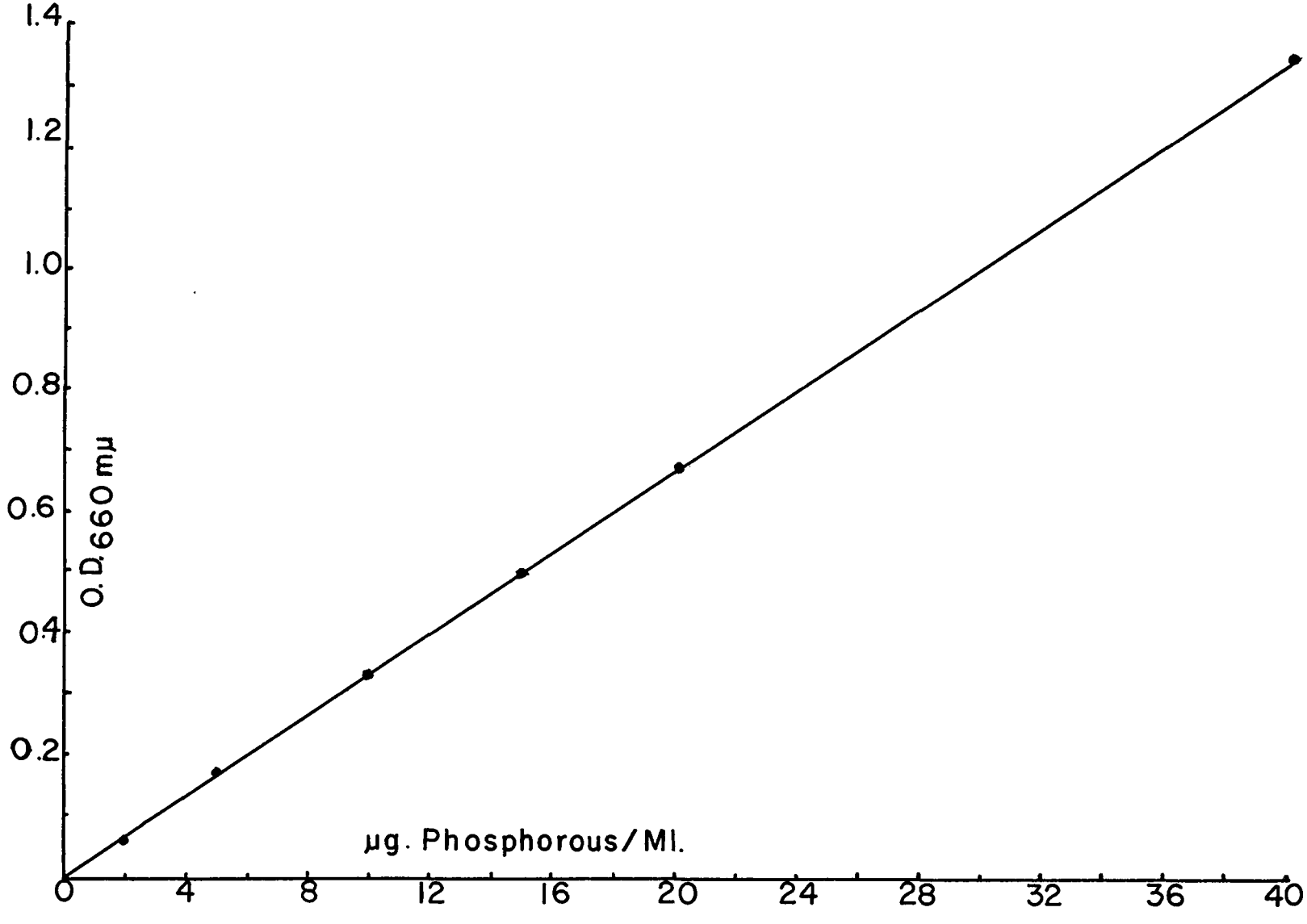

1.4
1.2
1.0
0.8
0.6
0.4
0.2
O.D. 660 mμ
μg. Phosphorous/Ml.
0
4
8
12
16
20
24
28
32
36
40

Fig. 4. P. rigidum A plasmodium growing on solid media of Henney and Henney (19). The Petri dish shown is 90 mm in diameter.

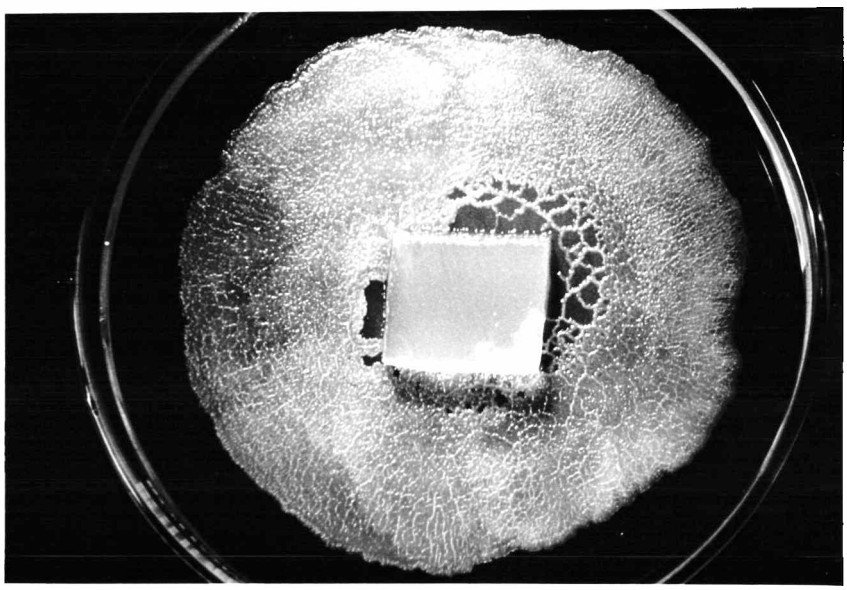
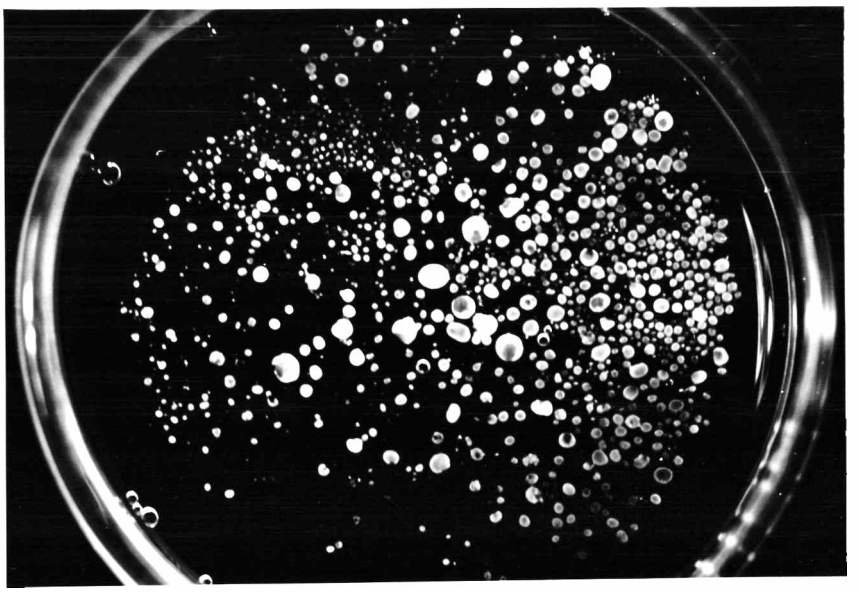


Fig. 5. Photograph A shows P. rigidum A microplasmodia grown in the liquid media of Henney and Henney. The sample was removed and placed in a 50 mm Petri dish for photography.

Photograph B shows the same sample at a 2X higher magnification.

A.



B.

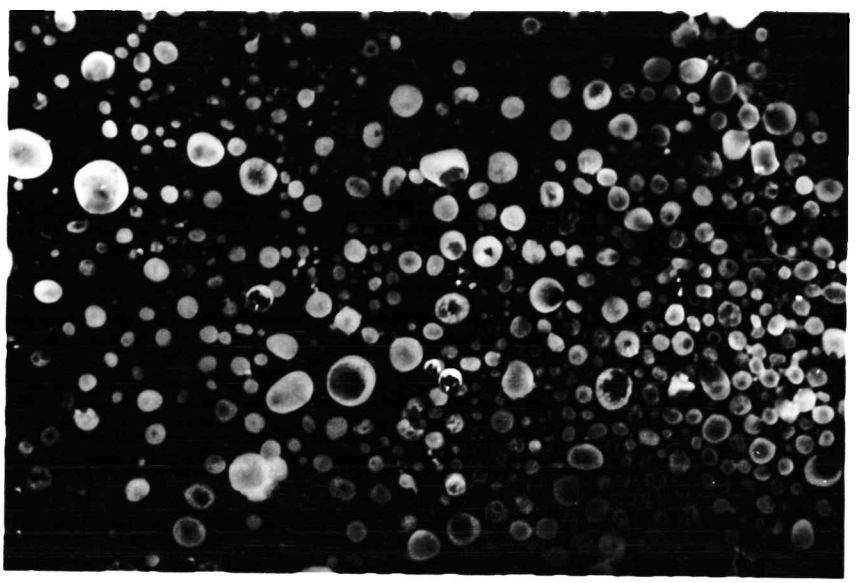
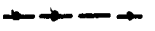


TABLE 1. RESULTS OF THE GROWTH STUDY ON P. RIGIDUM A IN mg /50 ml MEDIA

Day	Dry wt.	% change	Protein	% change	RNA	% change	Protein % dry wt.	RNA % dry wt.
0			0.019					
3	7.0		0.600		0.059		8.57	0.842
4	6.5	- 7.14	0.345	- 42.5	0.124	110.2	5.31	1.91
5	15.5	138.46	0.965	179.71	0.252	155.65	6.23	1.63
6	14.5	- 6.45	2.30	138.34	0.553	119.44	15.86	3.81
7	14.5	0.0	3.55	54.35	0.610	10.31	24.48	4.21
8	15.25	5.17	3.90	9.86	0.660	8.20	25.57	4.33
9	24.5	60.66	8.35	114.10	1.02	54.55	34.08	4.16
11	71.0	189.8	40.5	385.03	4.55	346.08	57.04	6.41
13	85.0	19.72	51.0	25.93	8.33	83.08	60.0	9.8
15	91.5	7.65	30.5	- 40.20	7.20	- 13.57	33.33	7.87

Fig. 6. The growth of P. rigidum A with respect to the following:

Dry weight 

RNA 

Protein

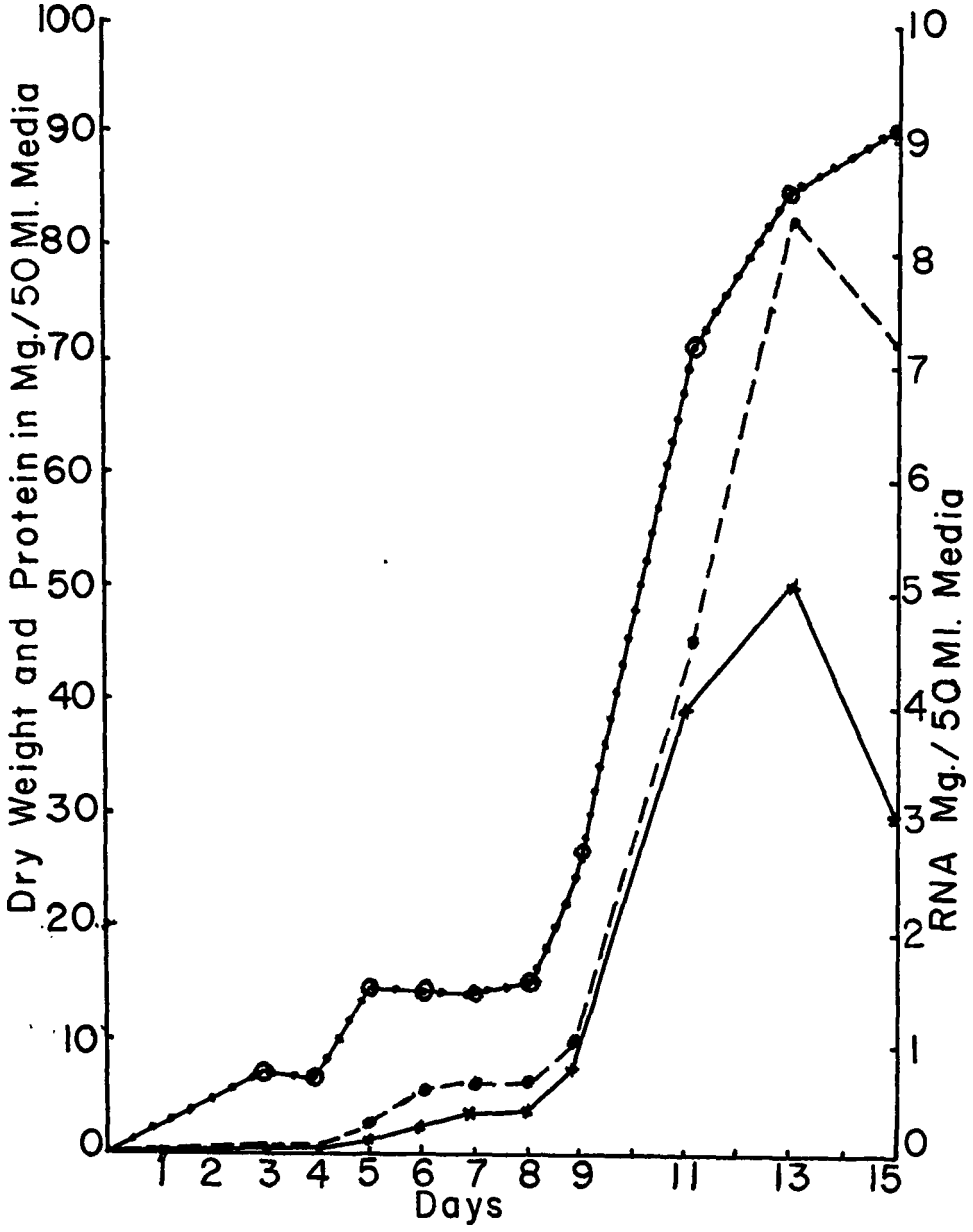

Dry Weight and Protein in Mg./50 Ml. Media
RNA Mg./50 Ml. Media
Days
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5
6
7
8
9
11
13
15

TABLE 2. COMPOSITION OF P. RIGIDUM YIELD/50 ml MEDIA

Component	Mg.	Percentage
Wet weight	945.0	
Water	860.0	91.0 (Wet wt.)
Dry weight	85.0	9.0 (Wet wt.)
Protein	51.0	60.0 (Dry wt.)
RNA	8.3	9.8 (Dry wt.)

Sedimentation Values of the Ribosomes

The study of the ribosomes proved somewhat difficult. The S_{20} results on the initial extracts in each buffer type are shown in Table 3. The largest peak averaged S_{20} 216.7. The other four peaks represented ribosomal material present at a very low concentration. They required the bar angle to be approximately 13 - 28 in order to raise the peaks to a reasonable 0.2 - 2.0 cm height above the baseline of the photos.

One of the better ultracentrifuge Schlieren patterns is shown in Figure 7. There the single ribosome peak with an S_{20} of 76 is rather prominant. The smaller peak of S_{20} 115 precedes it and corresponds to values assigned to a dimer (20).

An initial extract made in buffer with no magnesium added is shown in Figure 8. The small peaks corresponding to the subunits are visable. Their S_{20} values are 37.6 and 57.5.

Ribonuclease A added to the extracts did not break up the large initial peak that was found in all runs. Since polyribosomes are broken down into individual ribosomes under these conditions (20), the large peak must be aggregated ribosomes. Ultracentrifugation analysis using absorption optics also showed the large peak, and gave nearly the same S_{20} value. This proved that the peak absorbs in the ultra-

TABLE 3. AVERAGE SEDIMENTATION VALUES OF THE RIBOSOMES IN DIFFERENT BUFFERS

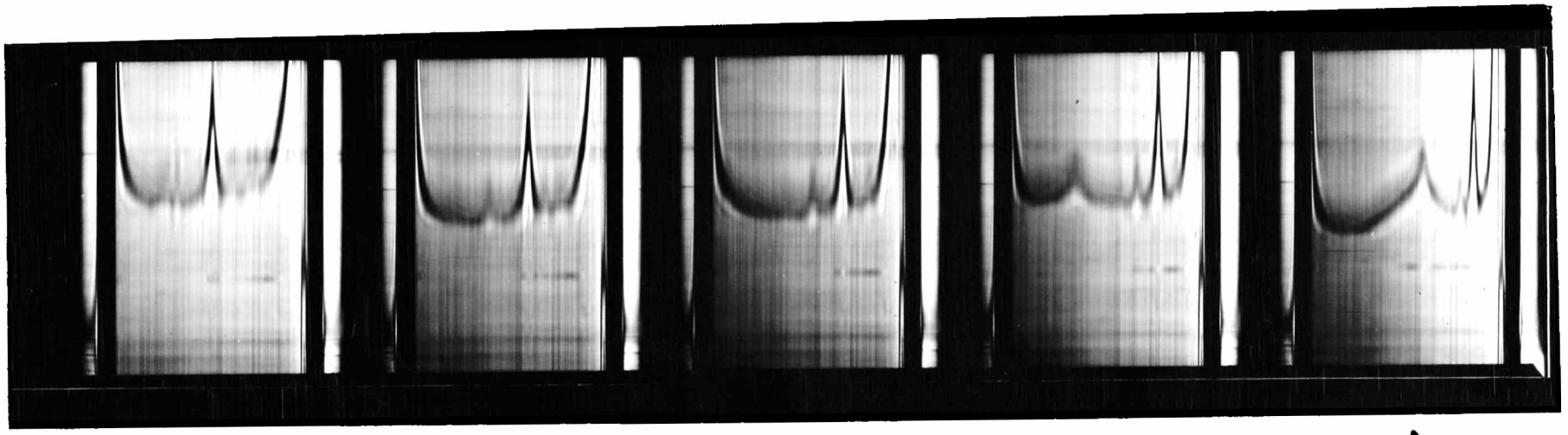
Buffer	X_1	X_2	X_3	X_4	X_5
A(1)			80.82	130.91	203.82
B(2)			75.12	114.41	229.61
C(1)			71.53	103.80	250.60
D(1)	37.85	55.53	74.41		192.96
E(3)	37.33	58.51	75.74	111.20	206.59
E*(4)		58.30	75.40		
Average	37.59	57.45	75.50	115.08	216.70
S^o_{20}	38.89**	59.44*	79.60	119.06*	

*With 0.2% Brij 58 added.

**Approximate values determined as described on page 18.

(The letters correspond to the buffers listed on p. 15. The number in parenthesis refers to the number of runs averaged.)

Fig. 7. Schlieren profile of ribosomes in initial extract of P. rigidum A. The tallest peak belongs to the 80 S class. The small preceding peak corresponds to a dimer. Photograph A corresponds to time zero.



A.

Fig. 8. The Schlieren profile of P. rigidum A ribosomes in the presence of a low magnesium concentration. The two small peaks trailing the 80 S peak are subunits. Two sample centrifugations are shown.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violet region.

Since aggregation can be due to extraneous proteins or membranes, the ribosomes were extracted under a 0.25 M potassium chloride concentration with 0.2 percent Brij 58 added. The single ribosome peak did show a slight increase, but the largest peak did not seem to decrease at all. Also, the ribosomes thoroughly washed for chemical analysis were centrifuged. The usual Schlieren profiles remained, except that the subunit peaks also appeared following these manipulations.

Since magnesium has been involved in aggregation (28, 39), the runs made in the absence of magnesium in the extraction buffer were compared with the highest magnesium concentration used. In the first case, the S_{20} of the large peak was the lowest value obtained, 193. In the latter case, the magnesium concentration was 0.05 M. The S_{20} value here was the highest one obtained, 250.6. The ribosome concentration during the two runs was similar as is shown by the O.D. 260 mμ. This was 29.3 and 26.0 respectively. However, no definite conclusions can be drawn other than the fact that the ribosomes do easily and consistently aggregate.

Overall, the single ribosome peak showed up better when the extraction buffer was 0.01 M Tris - HCl, pH 7.4 made 0.01-0.005 M in magnesium acetate, plus 0.25 M potassium chloride. The S_{20} value of the single ribosomal unit was corrected for

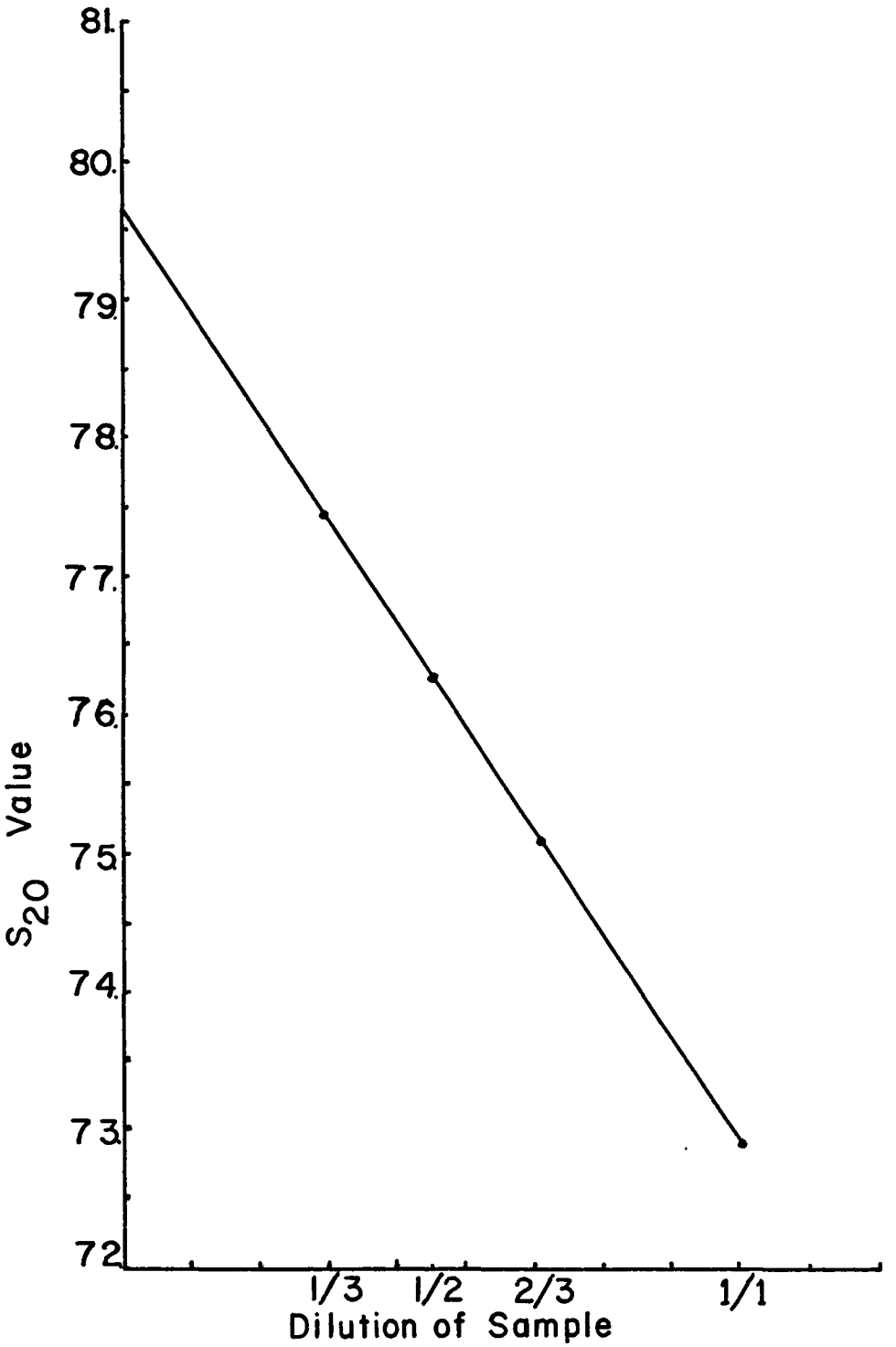
concentration as described. The plot after correction for deviation by the method of least squares is shown in Fig. 9.

The subunits and dimers were not present in sufficient quantities to permit a direct determination of the S^{o} value. Their peaks in the most concentrated extract were about the size of the 79.6 S peak in the most dilute concentrations at which it was visable with Schlieren optics. The difference between the average 76.94 S_{20} value of the two most dilute concentrations, and the final 79.6 S^{o}_{20} value is 3.46 percent. This percentage increase for the single ribosome peak was applied for a rough estimation of the S^{o}_{20} value of the two subunits and the dimer. These were 38.89, 59.44 and 119.06 respectively.

Chemical Analysis of the Ribosomes

The ribosomes for chemical analysis were extracted in the presence of Brij 58 to separate the ribosomes from the membranes of the microsome (15). They were washed in 0.25 M sucrose and 0.25 M potassium chloride in order to remove residual membranes and any contaminating proteins which are released under these conditions. The ribosomes were also subjected to low ionic strength and high pH buffers to remove proteins which are subject to release under these conditions (39). The ribosome pellet thus obtained had the proper gelatinous consistency, but could not be freed com-

Fig. 9. The extrapolation to zero concentration of the S_{20} values of ribosomes from P. rigidum A.


81.
80.
79.
78.
77.
76.
75.
74.
73.
72
S_{20} Value
1/3
1/2
2/3
1/1
Dilution of Sample

pletely from a yellow brown color characteristic of this organism. The Schlieren profile during analytical ultracentrifugation showed that subunits, single ribosomes, dimers, and aggregated ribosomes were present as usual.

Chemical analysis was made on the entire ribosome suspension and the results related back to 1 ml of the ribosome suspension. Before aliquots were withdrawn for analysis, care was taken to mix the suspension well. Completely separate analyses were made for each component in duplicate or more, and the results agreed well.

The concentration of protein/ml was 4.40 mg, and the phosphorous was 146 µg/ml. The O.D. 260 mµ of the hydrolyzed ribosomes was 61/ml. There were 606 µg of D (-)ribose/ml. The calculations of the RNA are as follows:

$$\frac{146\ \mu g\ \text{phosphorous} \times 100}{8.955\% \times 1000} = 1.63\ \text{mg RNA/ml}$$

$$\frac{606\ \mu g\ \text{D (-)ribose} \times 100}{43.847\% \times 1000} = 1.38\ \text{mg RNA/ml}$$

$$\frac{\text{O.D. } 61.}{36.558} = 1.67\ \text{mg RNA/ml}$$

The percent RNA and protein by each method, the average percentages, and the protein/RNA ratio are given in Table 4.

Base Composition Studies

The base ratio of ribosomal and total RNA was deter-

TABLE 4. COMPOSITION OF THE RIBOSOMES WITH RESPECT TO RNA AND PROTEIN

	% RNA	% protein	$\frac{\text{protein}}{\text{RNA}}$
Phosphorous	27.03	72.97	2.70
Orcinol	23.88	76.12	3.19
O. D. 260	27.51	72.49	2.64
Average	26.14	73.86	2.83

mined as stated in the Methods section. The O.D. 260 mμ graphs, and the O.D. 280/260 ratios proved very useful in deciding which fractions were to be included with each nucleotide peak. The work done with the commercial nucleotides and the E. coli B is shown in Table 5. Also included is a published total RNA nucleotide composition of E. coli B (37).

The commercial nucleotide recovery on C and G should have been higher due to the distinct peaks found on the graphs. However the nucleotides used were not of the highest purity, so corrections were made for this. More emphasis was placed on the E. coli B results because this tested the entire procedure, including the sample preparation process. Here the results were much better, especially when one considers the percent deviation found by the other investigators. Values for C and G were not as poor as was first indicated by the nucleotide recovery experiment.

The results of eight analyses using six different widely spaced samples of P. rigidum A total RNA are shown in Table 6. The average percent composition and deviation are given. Table 7 gives the individual analysis results and the averages using ribosomal RNA from P. rigidum A. The figures are from 5 separate determinations using 3 different samples. Also shown are the base ratios for P. polycephalum ribosomal RNA (12).

TABLE 5. RESULTS OF THE CONTROL CHECKS ON THE METHOD USED TO STUDY THE BASE COMPOSITION OF RNA

Source	C	A	U	G
E. coli B total RNA control	20.4	24.1	21.7	33.8
E. coli B total RNA (37)	21.0	27.0	17.0	35.0
Commercial nucleotide % recovery	92.9	100.0*	99.5	91.5 *

*Corrected for the purity of the nucleotide.

TABLE 6. NUCLEOTIDE COMPOSITION OF TOTAL RNA OF P. RIGIDUM GIVEN AS MOLES/100 MOLES OF IDENTIFIED NUCLEOTIDES

Experiment No.	G	Deviation	C	Deviation	A	Deviation	U	Deviation
1	32.42	1.25	19.76	0.94	24.25	0.16	23.57	0.15
2	34.57	0.90	19.62	0.80	22.76	1.33	23.05	0.37
3	34.10	0.43	18.66	0.16	23.55	0.54	23.69	0.27
4	33.09	0.58	18.73	0.09	24.43	0.34	23.75	0.33
5	34.76	1.09	17.61	1.21	23.52	0.57	24.11	0.69
6	35.04	1.37	18.01	0.81	23.70	0.39	23.25	0.17
7	33.85	0.18	18.72	0.10	24.39	0.30	23.04	0.38
8	31.55	2.12	19.48	0.66	26.09	2.00	22.88	0.54
Average	33.67	0.99	18.82	0.60	24.09	0.70	23.42	0.36

TABLE 7. NUCLEOTIDE COMPOSITION OF RIBOSOMAL RNA OF *P. RIGIDUM* A GIVEN AS MOLES/100 MOLES OF IDENTIFIED NUCLEOTIDES

Experiment No.	G	Deviation	C	Deviation	A	Deviation	U	Deviation
1	33.10	1.48	16.96	2.37	26.34	0.09	23.60	0.80
2	30.45	1.17	19.86	0.53	26.44	0.19	23.25	0.45
3	30.38	1.24	21.12	1.79	26.99	0.74	21.51	1.29
4	31.07	0.55	20.24	0.91	26.27	0.02	22.42	0.38
5	33.09	1.47	18.46	0.87	25.21	1.04	23.24	0.44
Average	31.62	1.18	19.33	1.29	26.25	0.42	22.80	0.67
Physarum Polycephalum[a]	31.6		22.8		23.8		21.9	

[a]The base composition of *P. polycephalum* ribosomal RNA is included for comparison (12).

Table 8 gives the G + C value, purine/pyrimidine ratio, A + U/G + C ratio, and the ratio of the 6-aminonucleotides/ 6-keto nucleotides. Also, the average nucleotide molecular weight, the percentage of D (-) ribose, and the expected percent phosphorous are shown for both total and ribosomal RNA.

Purified RNA

Purified RNA was obtained only if the procedures described were used. The results of the sedimentation studies in the two buffers are given in Table 9. The best results were obtained in the acetate buffer. The RNA dissolved in the phosphate, EDTA buffer gave lower S values. This same RNA with the buffer changed to the acetate type, gave the original higher S values. The S values of the boundaries did not decrease after treatment with deoxyribonuclease. This is also shown in Table 9. The slight increase in S value may be attributed to the increased magnesium chloride concentration in the buffer (38).

Positive prints of the photographs made during a typical centrifugation are shown in Figure 10. Time zero is in the lower left corner. Time increases from bottom to top. The picture at the right is a continuation of the left. The three separate RNA bands are shown more clearly in Figure 11, which is an analytrol scan of the absorption photograph.

TABLE 8. CALCULATIONS AND COMPARISONS MADE FROM THE RNA BASE COMPOSITION STUDIES

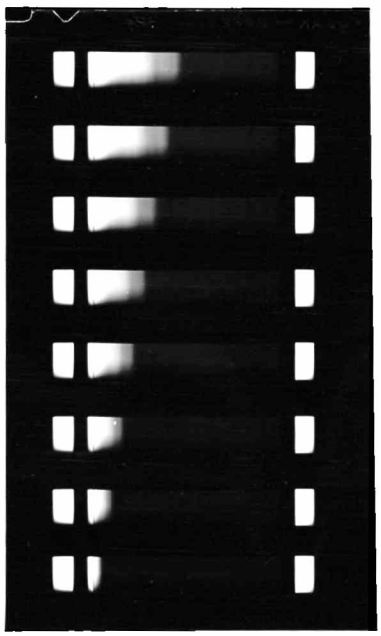
Type of comparison	Source of RNA		
	P. rigidum total RNA	*P. rigidum* r-RNA	*P. polycephalum* r-RNA (14)
Guanylic & cytidylic acids	50.95	52.49	54.40
Purines/pyrimidines	1.37	1.37	1.24
$\frac{\text{6-amino-nucleotides}}{\text{6-keto-nucleotides}}$	0.96	0.91	0.84
$\frac{\text{Adenylic + uridylic}}{\text{guanylic + cytridylic}}$	0.84	0.75	0.87
Average nucleotide mol. wt.	342.699	342.390	342.110
% D(-) ribose	43.808	43.847	43.88
% phosphorous	9.040	9.048	9.055

TABLE 9. S^{o}_{20} VALUES OF PURIFIED TOTAL RNA FROM P. RIGIDUM

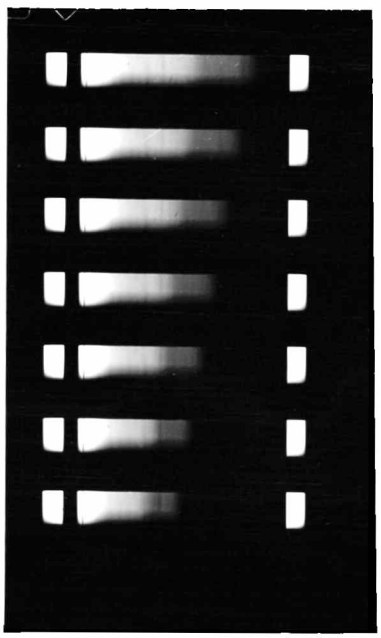
	X_1	X_2	X_3
0.01 M sodium acetate pH. 4.6 0.10 NaCl			
150 µg /ml	4.770	18.168	27.088
75 µg *	5.411	16.743	26.873
75 µg		17.785	27.455
37 µg		18.209	25.688
Average	5.091	17.726	26.776
75 µg after deoxyribonuclease treatment		19.72^{0}	28.81^{0}
	X_1	X_2	X_3
0.017 molal potassium phosphate pH. 6.8 0.01 EDTA			
100 µg /ml		14.385	21.874
50 µg	3.458	15.334	22.388
50 µg	4.821	17.488	22.329
Average	4.14	15.736	22.197

*RNA reprecipitated from the phosphate buffer.

Fig. 10. Analytical ultracentrifuge absorption photographs of purified P. rigidum A total RNA. The lower left corner of photograph A corresponds to time zero. Time increases by 4 minute intervals from bottom to top. Photograph B at the right is a continuation of the left.

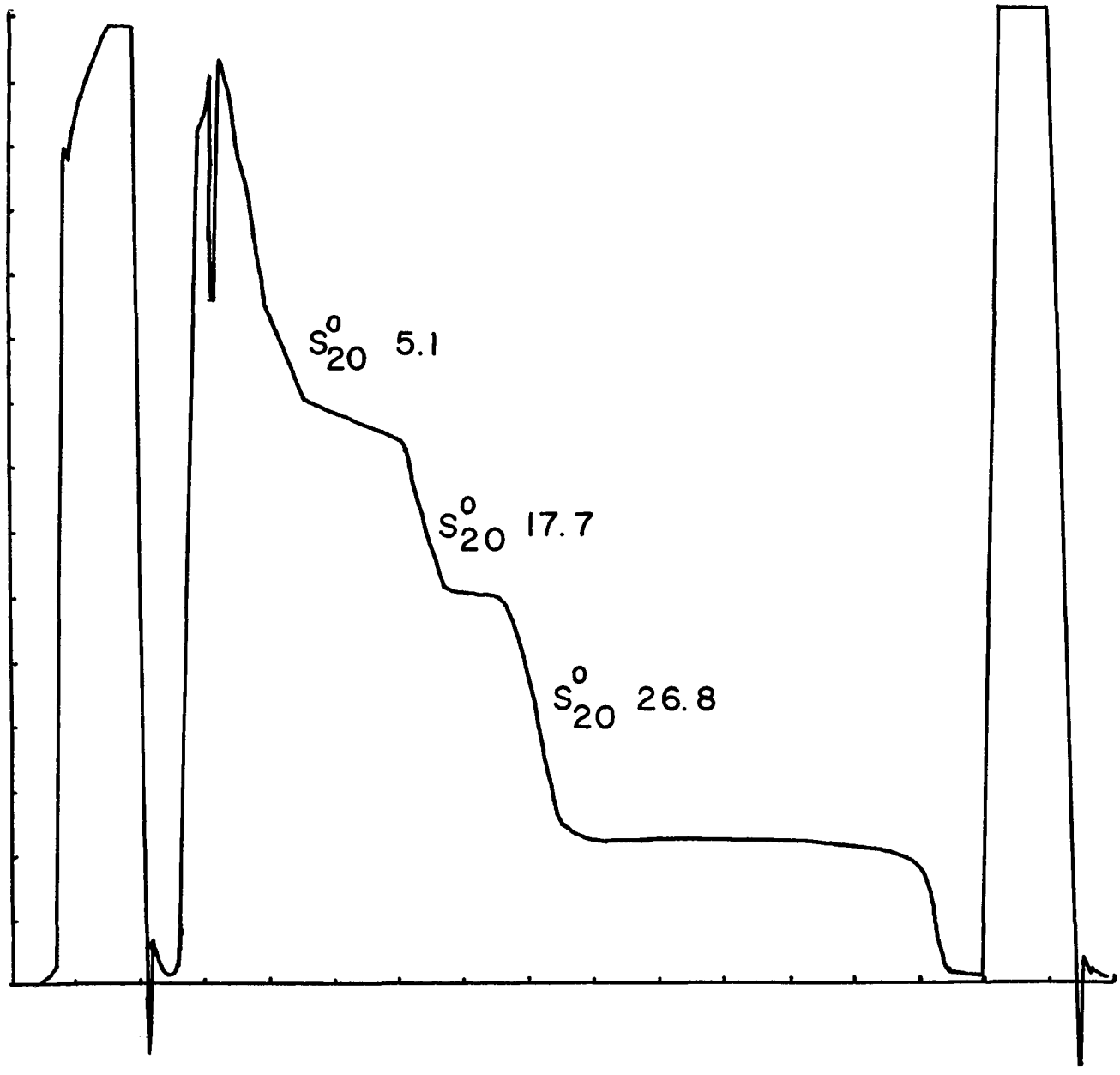


A.



B.

Fig. 11. An analytrol scan of the optical density changes in the absorption photograph of purified RNA.


S^{o}_{20} 5.1
S^{o}_{20} 17.7
S^{o}_{20} 26.8

Chemical and O.D. Characterization of Purified RNA

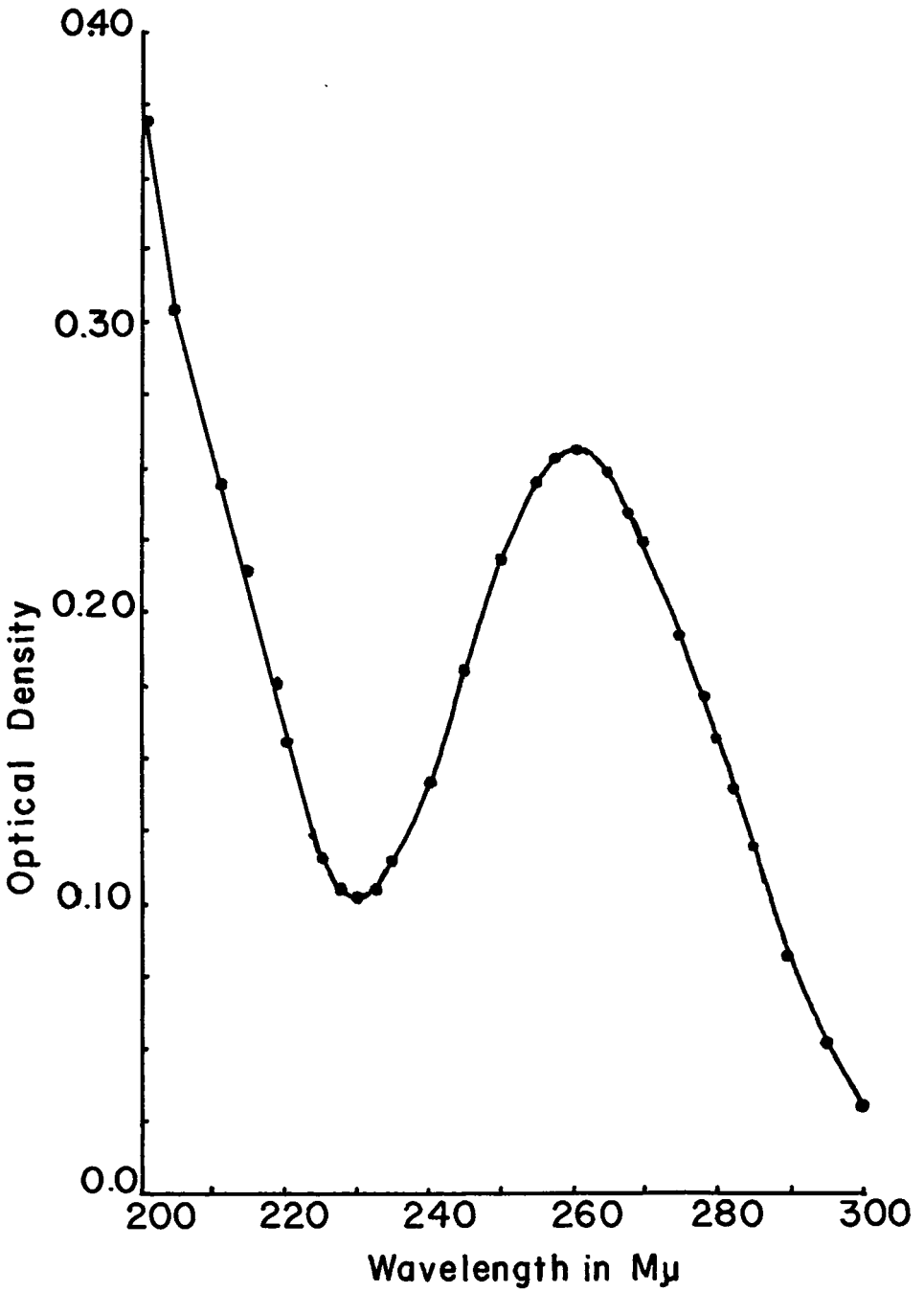
The RNA which had never been in the phosphate buffer was chosen for further tests and characterization. The absorption profile of the unhydrolyzed RNA is shown in Figure 12.

The orcinol test on the RNA solution gave good results with no tint of brown color. The duplicate tests gave the identical values of 499 μg of D (-) ribose/ml of solution. The prehydrolyzed RNA sample was next subjected to the orcinol test to check for higher results here due to a possible incomplete hydrolysis in the first determination. The results showed that pre-hydrolysis was not necessary, because the D (-) ribose was completely measured under the conditions of the test. In fact, after the increased manipulation, the 423 μg figure obtained was 15 percent lower than the first. Using the value of 43.808 percent D (-) ribose in the RNA, as obtained from the base ratio data, the amount of RNA in solution was calculated to be 1.139 mg/ml.

The phosphorous test gave the value of 102 μg/ml. This value corresponds to 8.955 percent of the RNA.

The O.D. 260 μg of the hydrolyzed RNA was 41.64/ml. This, divided by the actual RNA concentration, gives an extinction coefficient of 36.558/mg hydrolyzed RNA/ml of solution. The unhydrolyzed RNA had an O.D. of 25.6/ml when

Fig. 12. The absorption of purified RNA as a function of wavelength. The peak is at 260 mμ.


0.40
0.30
0.20
0.10
0.0
Optical Density
200
220
240
260
280
300
Wavelength in Mμ

read at 260 mμ. This calculates to be 22.476/mg unhydrolyzed RNA/ml of solution.

Part IV

DISCUSSION

Growth Study

With a small inoculum of 19 µg/50 ml of media, the normal growth curve showed the greatest increases between days 8-11. Here dry weight, RNA, and protein increased at the average rate of 128% per day. Between days 11-13, the rate of increase slowed to about 32% per day to give a maximum yield on day 13. After this point, the microplasmodia visually began to deteriorate. By day 15, the RNA and protein had decreased by 17%. The dry weight however did not drop at all. This may have been due to increased slime production.

The brown tint to the orcinol test could be due to several substances which interfere with the orcinol reaction (14). Therefore, the RNA values reported may be somewhat higher due to this background color. The profile of the protein curve coincided with the RNA curve, so this did not hinder selection of the proper point of harvest. By increasing the inoculum size, the day of maximum yield could be shortened to 7 days. This technique was used for all other harvests.

Day 13 was at the peak of the growth curve and was used for calculation of the RNA and protein as a percent of

dry weight. The percent protein and RNA were higher at the peak of the curve than at any other time. The 60% protein value is rather high. The averages for bacteria, yeasts, and molds are 60, 50, and 40%, respectively (27). The range of protein values found in yeasts and molds is 14 - 75%. The RNA value is somewhat higher than that of P. polycephalum, where the RNA/protein ratio is 0.08 (6). In P. rigidum the value is about twice that. The 91% water content is above the 69-89% range found in yeasts and molds (27).

Ribosome Sedimentation Studies

The sedimentation studies on the ribosomes proved rather difficult due to aggregation. In all cases, the majority of the ribosomal material sedimented in the peak which was about S_{20} 217. The centrifuge cell cleared rapidly and left up to four peaks in low concentration. The S^{o}_{20} 119 peak could be a polyribosome dimer (20), but until it is obtained in large enough quantities to quantitate the area under the peak before and after ribonuclease exposure, no definite conclusions can be drawn. The value of the 79.6 S^{o}_{20} peak represents that of single ribosomes in other eucaryotic organisms (46). This agrees with the value found in P. polycephalum.

There is some magnesium dependence in the binding of the subunits because these were only obtained in low magnesium

concentrations. This is in accordance with reports in earlier literature (36, 39). The 37.59 and 57.45 S_{20} values for the subunits are in perfect agreement with those expected for an 80 S ribosome (39). This contrasts with the values of 50 and 30 S which were assigned to P. polycephalum using sucrose density gradient centrifugation (32).

The chemical analysis of the ribosomes was another point of difference. P. polycephalum ribosomes had 47.9% RNA and 52.1% protein as calculated from its ribosomal RNA/protein ratio (32). This differs sharply from our values of 26.14% and 73.86% for RNA and protein respectively. The values for P. polycephalum were determined in a different manner (32, 34). The investigators prehydrolyzed with ribonuclease until 95-99% of the RNA was degraded. They then mildly acidified, centrifuged, and determined the amount of D (-)ribose in the supernatant. For their calculations, they applied a 2:1 conversion factor which doubled the ribose value (32). This factor is used because it is well known that hydrolysis of high molecular weight RNA with mild acid releases all purines and only a small fraction of the pyrimidines. It seems that prehydrolysis with ribonuclease could cause release of more than the expected amount of pyrimidines, if not all of them. Then application of the 2:1 correction factor would double the amount of RNA. Even if a factor should be used, a look at the base ratio of the

ribosomal RNA of P. polycephalum (12) gives a correction factor of only 1.8:1.

No conversion factors were applied to P. rigidum because a study of the purified RNA showed that the sample was completely hydrolyzed under our conditions of the orcinol test. Also the close agreement of the amount of RNA by three distinctly different methods reinforces our conclusions. However, it must be pointed out that of the three, the orcinol procedure gave an RNA percentage which was 4% lower than the other two, but application of a doubling correction factor would have caused a much larger discrepancy.

With regard to the protein analysis, the work with P. polycephalum was done with the biuret test which is specific for peptide linkages. The Lowry test may be subject to error because its sensitivity is not restricted to only peptide linkages.

The 2.8:1 protein:RNA ratio of P. rigidum A is much lower than the 2:1 - 1:1 ratio found in most eucaryotic organisms (36, 39). The washing procedure was very versatile and thorough. It seems unlikely that such a large difference could be attributed solely to contaminating proteins. If that is the case, however, the extraneous proteins are extremely well bound to the ribosomes.

RNA Base Composition Studies

The O.D. 280/260 ratios were important in calculating the amount of cytidylate eluted from the column. Cytidylate came off the column slowly after the initial peak, and when the next buffer was added to elute adenylate, a sharp spike came off adjacent to the adenylate peak. Fortunately the O.D. 280/260 ratios of the two are very different. Cytidylate is near 2.1 and adenylate about 0.395. The spike's ratio was about 1.9 and was thus included with cytidylate. This peak did not always show up. When it did, it varied from a very small rise to a peak which could change the value of cytidylate by several percent. As long as there was a uniform grouping of the O.D. 280/260 ratios, consistent results could be obtained whether the small spike was present or not.

The ribosomal RNA analyses occasionally showed the same spike and were handled the same way. The total and ribosomal RNA are of the G+C type. This is the usual case for most higher organisms (47). The ribosomal RNA base composition of P. rigidum compared to that of P. polycephalum shows much similarity. The main difference lies in an increased C and decreased A in P. polycephalum. This causes a corresponding change in the other ratios, but does not show up in the 6 amino/6 keto-nucleotide ratio.

The average nucleotide molecular weight, the percent phosphorous, and the percent D (-)ribose, are nearly the same in both total and ribosomal RNA. For example, the

percent phosphorous varies by only 0.008% between the two.

Purified RNA Sedimentation Studies

The purified RNA gave three distinct boundaries with the expected S values for ribosomal and soluble RNA. The 26.8 and 17.7 S°_{20} RNA's are the predominant types. This is obvious when one observes that these two species account for most of the ultraviolet absorption in the cell as shown by the Analytrol profile of the absorption photograph. This is in keeping with other organisms. P. polycephalum ribosomal RNA is 87% of the total cellular RNA (7). Other authors have found that the 28 and 18 S RNA correspond to ribosomal RNA (17,28). The 5.1 S°_{20} RNA is considered to be soluble or transfer RNA (30).

Chemical and O.D. Characterization of the Purified RNA

The ultraviolet absorption profile of the purified RNA fits that for the four major nucleotides of RNA (5).

The orcinol test on both prehydrolyzed and unhydrolyzed RNA gave essentially the same results. No correction factor was found to be necessary. The direct D (-)ribose value was used as a basis for further calculations. Any error in this first assumption would have shown up as unusual results later. The phosphorous in the solution was found to be 8.955% of this absolute value for RNA. That percentage is close to the 9% found by others (9).

The extinction coefficient of unhydrolyzed RNA also corresponds well with the values found by other investigators (20,44). The increase in O.D. 260 after hydrolysis of the RNA is expected (5,44).

Part V

SUMMARY

Physarum rigidum A grown on a liquid semidefined media was characterized as to its growth and partial chemical composition. It underwent a simple geometric increase in dry weight, RNA, and protein. By day 13, the peak of the curve was reached. At this point the organism contained 91% water. As a percentage of the dry weight, the protein and RNA were 60 and 9.8% respectively.

The ribosomes were shown to be of the 80 S class with an S^{o}_{20} value of 79.6. In low magnesium concentration, the ribosomes dissociated into 57.5 and 37.6 S_{20} subunits. This is typical of other eucaryotic organisms. A consistent 115.1 S_{20} peak corresponding to a polyribosome dimer was found. The ribosomes tended to aggregate and the majority of the ribosomes came off in a broad 216.7 S_{20} peak.

The washed ribosomes contained 26.14% RNA and 73.86% protein. This is a very high percent protein and possibly may be due to an unusually tightly bound extraneous protein on the ribosomes.

The base ratio of the total RNA shows it to be of the G+C type. This is the usual case with most higher organisms (47). The total RNA content of guanylic, cytidylic, adenylic and uridylic acids respectively is 33.67±0.99,

18.82±0.60, 24.09±0.70, and 23.42±0.36. The corresponding composition of ribosomal RNA is 31.62±1.18, 19.33±1.29, 26.25±0.42 and 22.80±0.67. The deviation in all cases averaged 0.8% or less.

Purified RNA gave S^{o}_{20} values of 26.8, 17.7 and 5.1. The first two correspond to ribosomal RNA and the latter to soluble RNA. This also is typical of eucaryotic organisms and in keeping with the 79.6 S^{o}_{20} value found for the ribosomes. The RNA had the expected percent phosphorous and D (-) ribose, with values similar to other RNA's studied.

The extinction coefficient calculated for the RNA also compares well with those found by others. In view of this information, I conclude that except for a low RNA: protein ratio in the ribosomal preparations, the ribosomes and RNA of P. rigidum A are typically eucaryotic in nature.

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