

ISOLATION AND ELECTROPHORETIC ANALYSIS OF  
NUCLEOLI, PHENOL-SOLUBLE NUCLEAR PROTEINS,  
AND OUTER CYST WALLS FROM *ACANTHAMOEBA*  
*CASTELLANII* DURING ENCYSTATION INITIATION

ROBERT W. RUBIN, MARY C. HILL, PAUL HEPWORTH,  
and JOYCE BOEHMER

From the Department of Biological Structure, University of Miami Medical School, Miami, Florida 33152 and the Division of Natural Sciences, New College, Sarasota, Florida 33578

ABSTRACT

A technique is described for isolating nucleoli from *Acanthamoeba castellanii*. Nuclei isolated by a modification of the technique of F. J. Chlapowski and R. N. Band (1971) are sonicated in a sucrose-Tris-MgSO<sub>4</sub>-KCl-Triton X-100 buffer and centrifuged on a linear sucrose gradient extending from 1.3 M to 1.5 M with a 2.6 M cushion, at 41,000 rpm for 90 min. The only apparent contaminants in the nucleolar preparation are outer cyst walls. A procedure is described for the isolation of chemically pure outer cyst walls, and a comparison of the proteins with the nucleolar preparation reveals that outer cyst walls represent negligible contaminants. The ultrastructure of these isolated nucleoli examined with transmission electron microscopy is found to be identical with that of nucleoli from whole cells, fixed in an identical manner. The 50 nucleolar proteins separated by SDS gel electrophoresis have been examined throughout the growth cycle of *Acanthamoeba* and into the start of induced encystment. No protein changes are observed until the beginning of encystment, at which time 10 protein bands disappear, 11 bands are observed to decrease, and 8 are seen to increase in concentration. Phenol-soluble proteins are extracted from the nucleolus which correspond to 29 of the 50 nucleolar proteins, with 17 of these proteins corresponding to nucleolar proteins that change at the onset of encystment. These nucleolar proteins are also compared with those of rat liver nucleoli by gel electrophoresis, resulting in the observation that extremely few protein homologies exist between the two. Numerous quantitative and qualitative changes in the gel pattern of phenol-soluble nuclear proteins during early and late log phase growth and the onset of stationary phase were also observed.

Information is now accumulating on the acid-soluble nucleolar proteins from a number of tissues (1, 22) and on acidic and SDS-soluble nucleolar proteins (7, 13, 16, 33). Extensive electrophoretic

analyses of rat liver nucleolar proteins have recently confirmed the great biochemical heterogeneity of these important organelles (22).

The nucleolus is known to be the site of synthesis

of ribosomal proteins as well as the location of ribosomal precursor assembly and processing (7). This subnuclear organelle is therefore a useful model for the study of biochemical events accompanying gene activation and ribosomal precursor assembly and processing.

This paper presents a method for the isolation of nucleoli and nuclear phenol-soluble proteins from a model system suitable for the study of both of these events. *Acanthamoeba castellanii* is a simple, single-celled eukaryotic organism which can be induced to undergo cellular differentiation involving the shut-down of its metabolism and the formation of resistant cysts with hard cellulose walls (19).

The proteins of the *Acanthamoeba* nucleolus were studied during log growth, stationary phase, and into the beginning of induced encystation to determine whether they change during periods of gene activation and/or deactivation. Since phenol-soluble nuclear proteins have been shown to change dramatically in content during differentiation in *Physarum* (15, 16), this fraction of the nuclear proteins was also analyzed on SDS polyacrylamide gels during induced encystation.

## MATERIALS AND METHODS

### Culture Methods

Axenic cultures of *A. castellanii*, strain *Mayorella palinensis* (MP), were grown in silicone-coated Erlenmeyer flasks in proteose-peptone-glucose medium (PPG) (2), using a New Brunswick Scientific Co., Inc. (New Brunswick, N.J.) controlled environment incubator shaker at 31°C and 180 rpm. Cells were grown to varying cell densities ( $6 \times 10^4$  to  $3 \times 10^6$  cells per ml) as determined by direct count with a hemacytometer. To induce cell differentiation and preparation for encystment, cells were transferred to and grown for 48 h in PPGF, reduced glucose medium (3, 4), after having reached a density of  $1.2 \times 10^6$  –  $1.5 \times 10^6$  cells/ml in PPG.

### Nuclear Isolation

Nuclei were isolated following a modification of the procedure of Chlapowski and Band (8). A Sorvall RC2-B automatic refrigerated centrifuge (Dupont Instruments, Sorvall Operations, Newtown, Conn.) and a Beckman Model L preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) were used in the following procedures. 3 liters of cells grown in PPG (or PPGF) were harvested directly and washed twice in 0.25 M STM (0.25 M sucrose, 0.005 M Tris, 0.002 M  $MgSO_4$ ) for 5 min, using a Sorvall GSA rotor at 1,000

rpm. The 25–35 ml of washed, packed cells obtained were resuspended to twice their volume in 0.25 M STM and homogenized with 30–40 up and down strokes (until ~90% of the cells were broken with nuclei still intact) in a 50 ml Potter-Elvehjem glass-Teflon homogenizer, model 74, 3/8 in drill at 1,000 rpm. The homogenate was brought to two times its volume with a 0.25 M STKM (STM with 0.025 M KCl) and centrifuged at 3,500 rpm for 15 min using a SS-34 rotor. The top light layer of the resulting biphasic pellet was carefully resuspended in 0.25 M STKM without disturbing the underlying dark pellet containing whole cells and cysts which was discarded. The suspension was washed three times in 0.25 M STKM by centrifugation at 2,000 rpm for 15 min, in the Sorvall SS-34 rotor. The washed pellet was resuspended in 3–4 ml of 1.3 M STKM, layered over a 4.5 ml continuous STKM gradient, extending from 1.3 M to 2.1 M, and centrifuged at 41,000 rpm for 45 min, in a Spinco 50.1 swinging bucket rotor. The resulting translucent pellet contains pure nuclei.

### Nucleoli Isolation Procedure

The sides of the tubes were wiped clean with tissue paper, and the nuclear pellets were suspended in 6 ml of 1.3 M STKM, 1% Triton X-100 and sonicated for 5 s at the maximum setting on a Savant Insonator, Model 500 sonicator (Savant Instruments, Inc., Hicksville, N.Y.). The sonicate was layered over a 4 ml continuous STKM gradient extending from 1.3 M to 1.5 M with a 2.6 M cushion and centrifuged at 41,000 rpm for 90 min in a Spinco 50.1 swinging bucket rotor. The pellet contains 95–99% pure nucleoli contaminated only by outer cyst walls (Fig. 1). The supernate was poured off and the sides of the tubes were wiped clean with tissue paper, care being taken to get all of the adhering contaminants from the 2.6 M – 1.5 M interface. The nucleoli were then ready to be prepared for electrophoresis (SDS-mercaptoethanol extraction), phenol extraction, and phase or electron microscopy as described below.

### Outer Cyst Wall Isolation

Cells grown as described above to a density of  $\sim 2.75 \times 10^6$  cells/ml were harvested at 1,000 rpm for 5 min in a GSA rotor. All subsequent operations were carried out at 0°C. The cells were washed twice with 0.01 M sodium phosphate buffer, 0.24 M sucrose, pH 7, suspended in 0.01 M sodium phosphate buffer, 0.24 M sucrose, 1% Triton X-100, pH 7 (NaPST), and homogenized 30 times (up and down), breaking up 95% of the cells in a 50-ml glass Teflon homogenizer at 1,000 rpm. The homogenate was spun at 3,500 rpm for 15 min in a SS-34 rotor. The pellet was resuspended in NaPST to 10 times its volume, sonicated for 2 min at the maximum setting, and centrifuged again at 3,500 rpm for 15 min in a Sorvall SS-34 rotor. This last step was repeated until all the nucleoli were destroyed as determined by examination

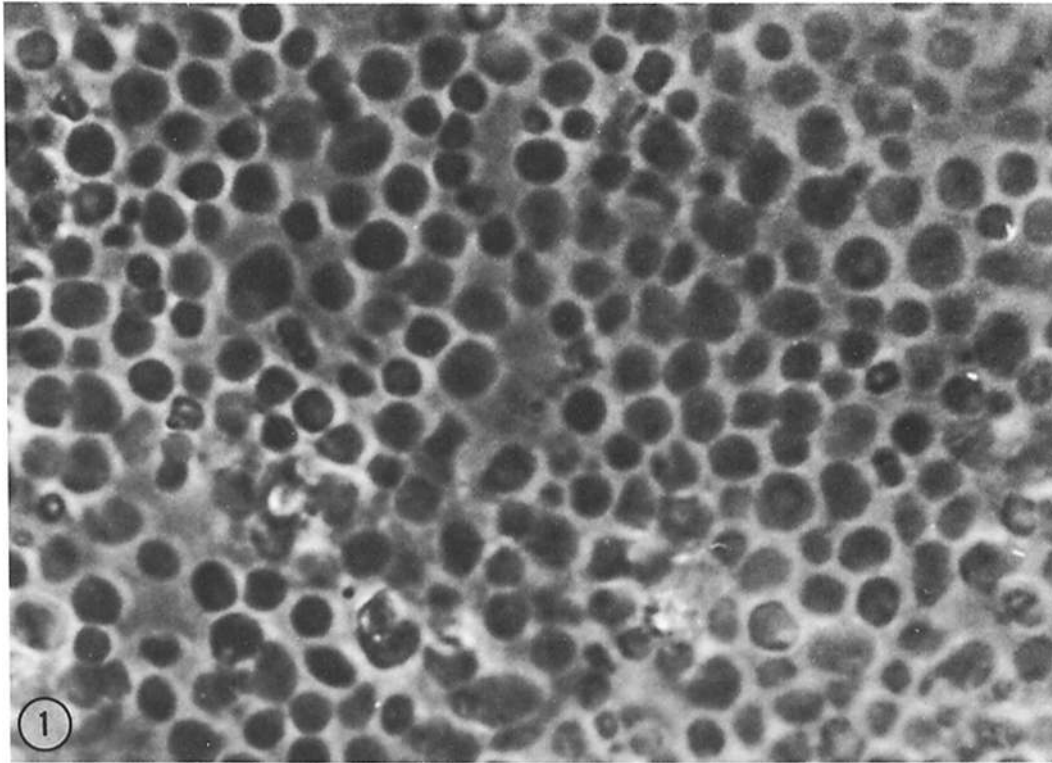


FIGURE 1 A light micrograph of isolated *Acanthamoeba* nucleoli. Note the presence of vacuoles in several of the nucleoli.  $\times 280$ .

under the phase microscope. The nucleoli-free pellet was then suspended in 2 ml of 1.3 M STKM (see above), layered over a 5-ml continuous STKM gradient extending from 1.3 M to 1.5 M with a 2.0 M cushion, and spun at 41,000 rpm for 90 min using a 50.1 swinging bucket rotor. The pellet containing outer cyst walls and a few whole cysts was resuspended in 1.3 M STKM, layered over a 1.3 M, 1.5 M step gradient and centrifuged in an IEC International clinical swinging bucket centrifuge, model CL, (Damon/IEC Div., Damon Corp., Needham Heights, Mass.) at 3,000 rpm for 25 min, to remove any remaining debris from the cyst walls. The pellet obtained was prepared for electron microscopy, phase microscopy, or gel electrophoresis as described below (Fig. 2).

#### *Isolation of Whole Cysts*

Cells grown as described above to a density of  $2.75 \times 10^6$  cells/ml were harvested at 1,000 rpm for 5 min in a GSA rotor. These cells were repeatedly washed with distilled water at 1,000 rpm for 5 min, using a GSA rotor until all cells had lysed, leaving only whole cysts in the pellet. This cyst pellet was layered over a step gradient of 1.3 M, 1.5 M sucrose and centrifuged in an IEC International clinical swinging bucket centrifuge, model

CL, at 3,000 rpm for 15 min to remove any cellular debris remaining in the cyst pellet. The pure cyst pellet thus obtained was prepared for gel electrophoresis as described below.

#### *Determination of Protein*

The protein content of each step of the nucleolar isolation procedure was determined by the method of Lowry et al. (17). Bovine serum albumin (BSA) was used as a standard. Aliquots for the protein determination were suspended in 0.25 M STKM solution and diluted with water.

#### *Extraction of Phenol-Soluble Nuclear and Nucleolar Proteins*

Phenol-soluble nuclear and nucleolar proteins were extracted according to the procedure of LeStourgeon and Rusch (16) involving the successive extraction of salt-and acid-soluble proteins, lipid extraction, and final phenol extraction of the residue. The proteins from 1.4 liters of  $1.5 \times 10^6$  cell/ml were precipitated from the phenol layer by diluting it to four times its volume with 100% ethanol and letting it sit at  $-20^\circ\text{C}$  for 3-5 days. The protein thus obtained was pelleted by centrifugation in a SS-34 rotor

at 16,000 rpm for 25 min using a Sorvall RC2-B automatic refrigerated centrifuge. The supernate was decanted, and 10 ml of 100% ethanol were added to the pellet which was allowed to stand at 0°C for 5 h and centrifuged again at 16,000 rpm for 25 min. The ethanol was poured off and the pellet heated in a water bath to dry it before preparation for gel electrophoresis as described below. This heating did not affect the protein gel patterns of the SDS-extracted final pellet.

### *Isolation of Rat Liver Nucleoli*

Rat liver nucleoli were isolated according to the procedure of Muramatsu and Fujisawa (18), where the pellets were prepared for gel electrophoresis as described below.

### *Gel Electrophoresis*

The polyacrylamide thin (16 × 12 × 0.08 cm) slab gel system of Laemmli (13) and O'Farrel et al. (21) was used, giving gels with an acrylamide concentration of 12% and a sodium dodecyl sulfate (SDS) concentration of 0.1%. Gels were made up no more than 72 h nor less than 12 h before use and were run at 12 mA through the spacer gel and at 15 mA in the lower gel.

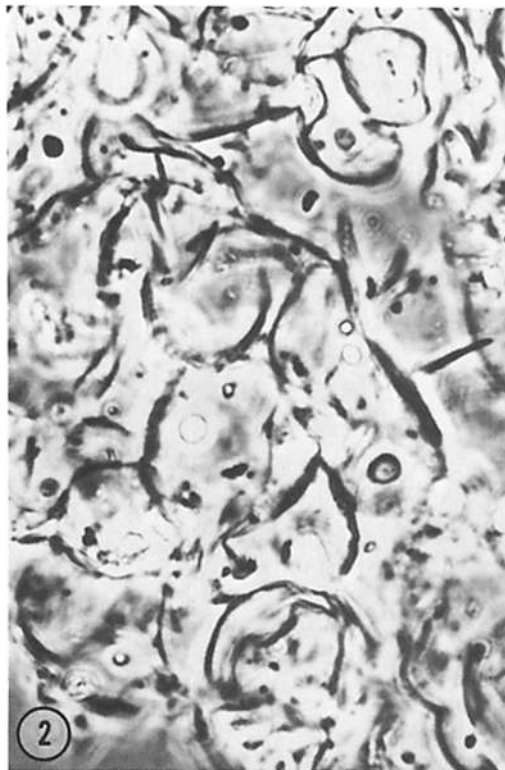


FIGURE 2 A light micrograph of isolated outer cyst walls. × 200.

Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, Mo., 23C-0950) (11) was used at a concentration of 1% in 25% trichloroacetic acid (TCA). Destaining took 1–2 days, employing changes of 8% acetic acid and mild shaking. Pellets obtained by the various procedures were dissolved in hot sample buffer, 10% glycerol, 5% mercaptoethanol, 2% SDS, 0.0625 M Tris, made up with bromophenyl blue as the tracking dye, before being applied to the gel.

### *Microscopy*

Phase optics were used for monitoring the isolation procedures and for obtaining light micrographs which were taken at a magnification of 40. The organelles examined were suspended in 0.25 M STKM. The approximate percentage of cell breakage after homogenization was determined by direct counting with a hemacytometer.

For electron microscopy, amoebae, nucleoli, and cyst walls were fixed in 3% glutaraldehyde in 0.01 M phosphate buffer (pH 7) for 1 h, washed with three changes of buffer over a 2-h period, and postfixed for 40 min in 3% osmium tetroxide. After dehydration the pellets were embedded in Epon, and sections were cut with a diamond knife and examined with a Philips-300 electron microscope.

### *Molecular Weight Determination*

The approximate molecular weight of the various peptides separated by SDS electrophoresis was determined from the linear relationship between electrophoretic mobility and the logarithm of the molecular weights of the following protein standards (30): chymotrypsinogen-A (beef pancreas), ovalbumin, gamma-globulin (human), albumin (bovine), cytochrome *c* (horse heart). Measurements were made from the middle of the band for each molecular weight standard and amoeba proteins. Band length measurements of the molecular weight standards indicated a range in molecular weight values of no greater than 5% of the known molecular weight of any given standard. 5% was then taken as the range in accuracy of the molecular weight values determined from gels of amoeba proteins.

## RESULTS

One of the most important physical factors of the isolation procedure was the sonication time. With too little sonication the nucleolar pellet was contaminated with nuclei; however, with too much sonication the nucleoli were broken up and completely dispersed. The amount of sonication described above resulted in disruption of over 99% of all the nuclei, with a minimum of nucleolar breakage. Removal of divalent cations by washing the nuclear pellet in STKM lacking divalent

cations or by adding EDTA or EGTA caused nucleolar disruption during sonication but did not free the nucleoli from the nuclei under milder homogenization conditions such as teflon pestle homogenization.

The relative amounts of protein present in the various stages of the isolation procedure are presented in Table I. The low percentage of protein present in the final nucleolar pellet is most likely due to loss during nuclear isolation and loss at the 2.6 M sucrose cushion where considerable nucleoli are trapped, as revealed by phase optics.

SDS gel electrophoresis revealed the outer cyst wall to contain nine proteins (see Fig. 7 E). All but one (at a mol wt of 13,000 daltons) of these proteins are in the medium-to-high mol wt range (45,000–90,000), with the major protein band of the outer cyst wall having a mol wt of approximately 70,000.

The only major contaminants (1–3% of the total particulate material) of the nucleolar pellet were outer cyst walls, as determined by phase and electron microscopy (see Fig. 2). These were determined to be outer cyst walls by their subfibrillar ultrastructure and by the fact that at this stage of culture age most cells possess only an outer cyst wall. This is consistent with recent work which indicates that the outer cyst wall is synthesized before the inner cyst wall (29). The amount of this contaminant increased as the cell density, age, and proximity to encystment increased. As can be seen in Fig. 7 E, however, none of the nine proteins of the cyst wall resolved by electrophoresis correspond to major bands in the nucleolar samples (Fig. 7); and, in the nucleoli, isolated from cells kept in glucose-starved media (PPGF), which had the greatest number of contaminating cyst walls, only two of the corresponding nucleolar bands increased in density (bands 18, 49). Only the increase in band 18 of this nucleolar sample could

be due to cyst wall contamination, as this is the major protein band of the outer cyst wall. The great increase in band density of nucleolar protein 49 could not be due to outer cyst wall contamination alone, because the nucleolar proteins corresponding to the other minor cyst wall bands did not increase to a similar extent. The other nucleolar bands which co-electrophoresed with the cyst wall bands underwent no change in density at all, there being even a slight decrease in the density of nucleolar band 20. Therefore, only the changes observed in nucleolar band 18 will be assigned to the possibility of cyst wall contamination during early encystment.

The outer cyst wall pellet used in these comparisons was contaminated by a few whole cysts. This contamination was negligible, however, as determined by a comparison of electrophoretic profiles of whole cyst preparations vs. outer cyst wall preparations (not shown) and by the fact that only one component constituted over 90% of the staining on the outer cyst wall gel (Fig. 7).

#### *Ultrastructure of the Nucleoli*

Phase and electron microscopy show that both the gross and fine structure of the isolated nucleoli are preserved by the isolation procedure (Figs. 1, 3, and 4). Both the intact and isolated nucleoli are observed to consist of two types of structural components, one granular and the other fibrillar, with each being segregated into distinct regions. The granular regions are located mainly along the periphery, with a small amount being near the center, of the nucleolus. The fibrillar regions are located in the center of the nucleolar mass and are surrounded by the granular areas. Electron-diffuse areas, variable in size and shape and confined mainly to the fibrillar regions, are observed and believed to be lacunar regions. These are constant features of the interphase nucleolus in whole cells and of isolated nucleoli and are characterized by the presence of chromatin material within their confines (9, 10). All of the observations are consistent with our present knowledge of the nucleolar ultrastructure of other organisms (7, 27).

#### *Comparison with Rat Liver Nucleoli*

As shown in Fig. 5 there is very little homology between the 42 rat liver and the 50 *Acanthamoeba* SDS-soluble nucleolar proteins visualized by SDS gel electrophoresis. It is of interest that the four major proteins of the rat liver nucleoli are of low

TABLE I

*The Relative Amounts of Protein Present in Various States of the Isolation Procedure for Acanthamoeba Nucleoli*

Step of procedure	Protein
	%
Whole cell homogenate	100
Homogenate after STKM washes	30.0
Nuclear pellet	1.8
Nucleolar pellet	0.1

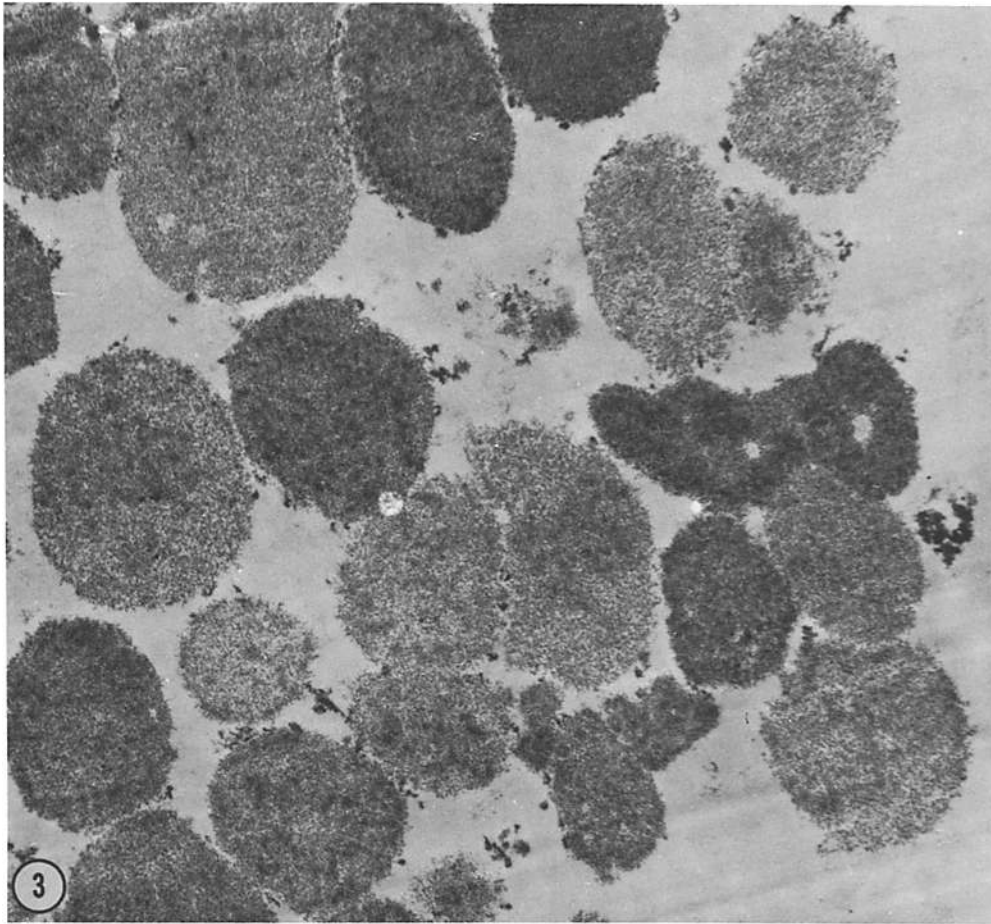


FIGURE 3 Electron micrograph of isolated nucleoli showing the purity of the preparation and the intact nucleolar ultrastructure.  $\times 9,500$ .

mol wt, 12,000–18,000 (perhaps histones), while the four major protein bands of the *Acanthamoeba* nucleoli are in the medium mol wt range of 32,000–65,000.

#### *Changes in Nucleolar Proteins During the Culture Growth*

No consistently observed changes occurred in the protein make-up of the *Acanthamoeba* nucleolus during the normal growth cycle (see Fig. 6 for growth curve) as indicated by samples A, B, and C of Fig. 7. After the cells have been in glucose-starved medium (PPGF) (3, 4), however, for 48 h, which stimulates them to start undergoing differentiation (5), many protein changes are observed (sample D, Fig. 7). Of the 50 bands

visualized by electrophoresis from nucleoli isolated from growing cells, only 37 are present in nucleoli of starved cells (bands 8, 15, 16, 21, 24, 35, 39, 41, 43 are not visible in PPGF nucleoli). Several high molecular weight components (bands 1, 3, 4, 6, 7, 9, 10, 11, and 12) and a few bands of medium and lower molecular weights (bands 20, 22, 28, 34, 36, 42) appear to decrease slightly in concentration at this time. The electrophoretic profiles of six protein bands (bands 14, 18, 23, 26, 40, 45) increase in concentration along with those of four other bands, mostly of low molecular weight (bands 31, 46, 48, 49), their concentration increasing greatly over the same protein bands from nucleoli isolated from growing cells.

Nuclei and nucleoli could not be isolated from cells placed in encystment media (20), due to the

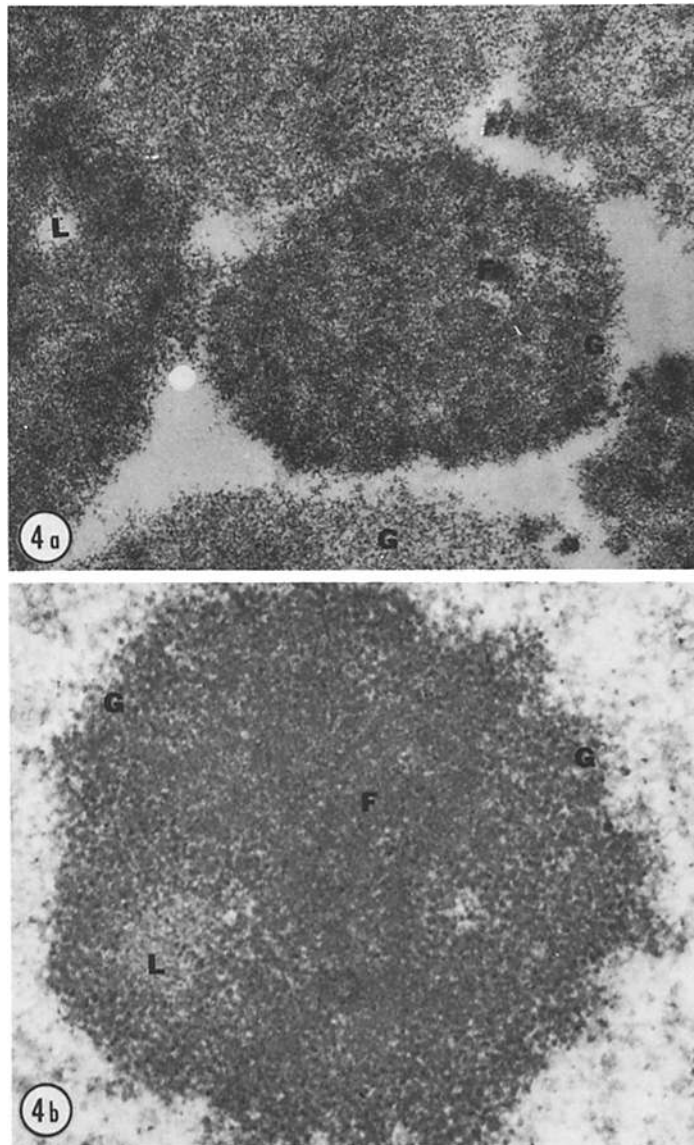


FIGURE 4 Electron micrographs of isolated nucleoli (*a*) and a nucleolus in the intact cell (*b*). Lacunar regions (*L*); granular regions (*G*); fibrillar regions (*F*). (*a*)  $\times 22,000$ ; (*b*)  $\times 33,000$ .

formation of both the inner and outer cyst walls. Once this happens, the cells cannot be lysed by homogenization procedures which preserve nucleolar structure.

#### *Characterization of Nucleolar and Phenol-Soluble Nuclear Proteins*

A total of 29 protein bands are extracted from nucleoli by the method of LeStourgeon et al. (16)

for extraction of phenol-soluble acidic proteins (see Fig. 8). The bulk of these proteins are in the medium to high molecular weight range, with relatively few in the lower molecular weight range. Of these protein bands, one (band 30) is increased in concentration in its extracted form as compared with its concentration in samples of whole nucleoli.

Of the 25 bands that disappear or decrease in concentration during the onset of encystment, 13 are phenol soluble (bands 8, 15, 16, 1, 3, 9, 11, 12,



FIGURE 5 An SDS polyacrylamide gel comparing SDS-mercaptoethanol-soluble rat liver nucleolar proteins (B) and *Acanthamoeba* nucleolar proteins (A). Note the striking lack of homology between the two.

34, 20, 22); and of the nine bands which increase in concentration, five are pheno-soluble (bands 23, 31, 40, 45, 46). Band number 29 was found to have the same electrophoretic mobility as actin, but constituted one of the less prominent components.

For unknown reasons, the resolution of bands from phenol extracts of whole nuclear preparations was considerably less clear than that of bands from nucleolar preparations with extensive band spreading. However, 15 components were consistently observed. A comparison of densitometric traces (Fig. 9) of phenol-soluble acidic nuclear proteins extracted from cells in early log phase growth (a) and from the PPGF starvation-induced stationary phase (b) reveals the overall changes in these proteins from widely separated stages in culture growth. Generally, the changes are characterized by a decrease in the number of protein species during the stationary phase, especially in the higher mol wt range from 46,000 to 100,000 daltons, as compared to those present during

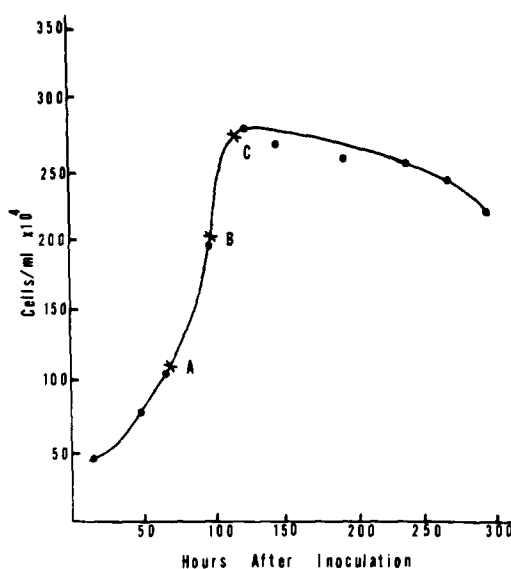


FIGURE 6 The growth curve of the *Acanthamoeba*. Samples A, B, and C (of Fig. 9) were harvested at the points indicated on the growth curve, representing cultures containing low, medium, and high densities of cells.

periods of active growth. There is a marked increase in the amounts of several lower mol wt proteins from 18,000 to 25,000 daltons, and the relative concentrations of the proteins of mol wt 43,000 and 56,000 have been reversed.

The heterogeneous protein composition at each of the observed stages of culture growth showed unique reproducible patterns which differed quantitatively and qualitatively from stage to stage. Some of the changes in nucleolar and nuclear protein molecular weight profiles could be due to changing nuclear protease levels during encystment. At the lowest cell density (A, Fig. 6), nine major proteins were observed. As the cell density increased (B, Fig. 6) more proteins appeared; there were three in the mol wt range 48,000 to 58,000, a high mol wt protein at 100,000, and the large protein aggregate at 24,000–28,000 became three separate components. This pattern was observed until the cell density reached the level of approximately  $2 \times 10^8$  cells/ml (C, Fig. 6). At this stage the aggregate reappeared, a new component at 19,000 appeared, and two high molecular weight proteins at 73,000 and 80,000 daltons disappeared. Several more proteins at 8,000, 10,000, 24,000–28,000, 49,000, 53,000, 90,000 and 100,000 daltons disappeared at 130 h (see Fig. 6). By 300 h during poststationary decline and during the



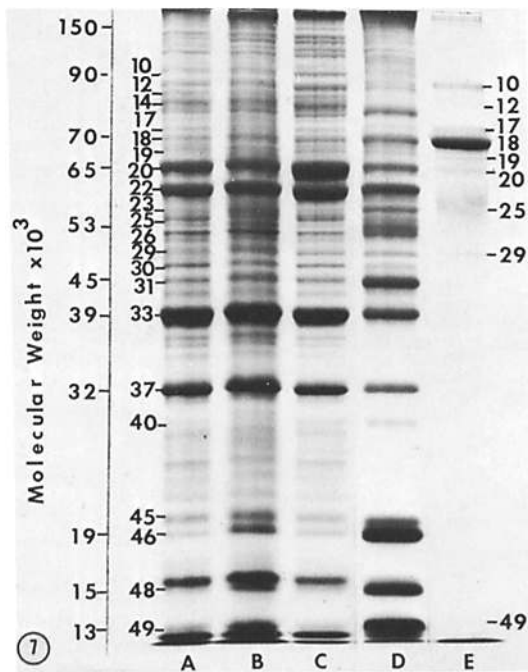


FIGURE 7 An SDS polyacrylamide slab gel of representative nucleolar samples from cells harvested during active growth at a density of 1,015,000 cells/ml (A); 1,980,000 cells/ml (B); 2,700,000 cells/ml (C); and nucleoli from cells kept in glucose-starved medium PPGF, which initiates the beginning of encystment (D). Sample E is outer cyst wall proteins. Note the concentration differences between the proteins extracted during active growth and those extracted during the initial stages of encystment. Also note the existence of only one major protein band in the outer cyst wall.

PPGF starvation-induced stationary phase (not shown), a major protein of mol wt 23,000 reappeared and the protein of mol wt 19,000 increased dramatically in concentration.

The quantitative changes in the proteins of mol wt 43,000 and 46,000, which were the most prominent bands on the electrophoretic profiles, are demonstrated by the densitometer traces shown in Fig. 9. As cell density increased, the higher molecular weight protein (left vertical line) steadily increased while the relative amount of the lower molecular weight protein (right vertical line) decreased.

#### Comparison of Phenol-Soluble Nuclear and Nucleolar Proteins

Comparison of the phenol-soluble acidic nuclear proteins and the phenol-soluble acidic nucleolar

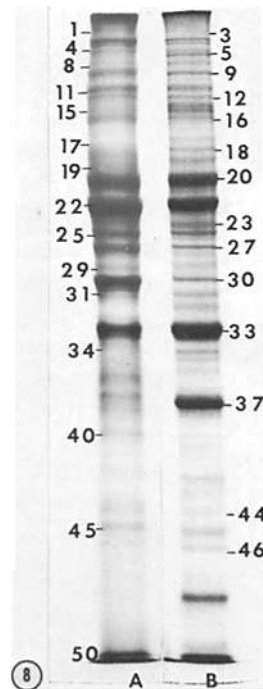


FIGURE 8 A gel comparing phenol-soluble nucleolar proteins (A) and total nucleolar proteins (B). Note the great number of nucleolar proteins that are phenol soluble. Also note that the bulk of the phenol-soluble acidic proteins are of medium to high molecular weight, with very few being in the low molecular weight range.

lar proteins which were resolvable on densitometer traces indicated that the lower mol wt component of 43,000 daltons is probably nucleolar in origin. The major protein components of mol wt 50,000, 52,500, and 57,000 also appear to be of nucleolar origin.

#### DISCUSSION

The nucleoli isolated from *A. castellanni* by the procedure presented in this paper, being pure and intact (see Fig. 1), can now be used in a number of studies involving cellular differentiation and genetic regulation. Although acidic nucleolar proteins have been analyzed by one-dimensional electrophoretic techniques for *Physarum polycephalum* (16), in which one protein change was observed during cellular differentiation, and for rat liver (33), to date there has been no information on whether or not any of the proteins extracted from the whole nucleolus change during differentiation. The present results demonstrate that certain nucleolar proteins isolated from *Acanthamoeba* appear in greater concentrations during differentia-

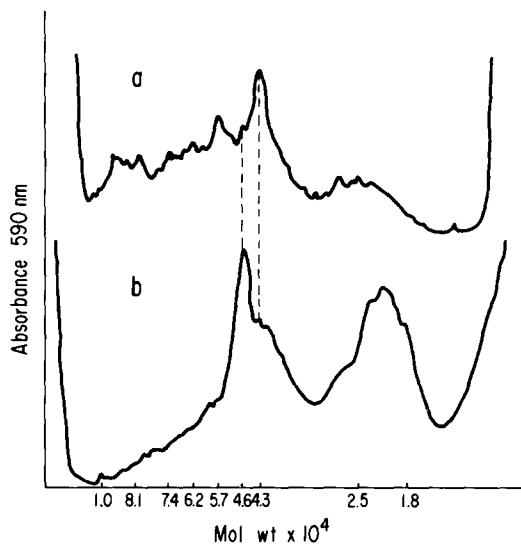


FIGURE 9 Abscissa: molecular weight  $\times 10^4$ . Ordinate: absorbance (590 nm). Comparison of densitometric traces of phenol-soluble acidic nuclear proteins extracted during (a) early log phase growth (point A, Fig. 6), and (b) after PPGF starvation. Note disappearance of high molecular weight proteins, the marked increase in the proteins of molecular weight near 23,000 daltons, and the quantitative differences in the proteins of mol wt 43,000 and 46,000 daltons.

tion while others disappear or are observed to be in lesser quantities at this time.

Research presently underway in this laboratory and elsewhere indicates that protein synthesis in *Acanthamoeba* continues through the induction phase and into the initiation phase (20) (first appearance of round forms) of encystment, at which time it is turned off. This is consistent with the findings presented here which show few qualitative or quantitative changes in nucleolar peptides until the first appearance of outer cyst walls and precysts (round forms).

It is of interest that 29 of the 50 nucleolar proteins resolved by gel electrophoresis are phenol soluble. This finding is consistent with previous reports that nucleolar proteins are largely acidic and/or phenol soluble in character (6, 27). Some of these acidic nucleolar proteins are thought to be enzymes involved in nucleolar metabolism and rRNA synthesis; some are ribosomal proteins destined to combine with rRNA (7, 27).

Since acidic nuclear proteins are thought to be involved in gene regulatory functions (15, 24, 25, 28, 32), it is possible that some if not all of the

acidic nucleolar proteins which change during cellular differentiation are involved in the masking or unmasking of nucleolar DNA coding for rRNA.

The observed result that phenol-soluble nucleolar protein band 30 is increased in its extracted form as compared with its concentration in samples of whole nucleoli could be due to the protein's having a very high solubility in phenol over the other proteins, or the protein's being less tightly bound in the nucleolus than the rest of the proteins extracted by this procedure.

Isolated whole (inner + outer) cyst walls of *Acanthamoeba* have been reported to be 33% protein, 33% cellulose, 6% lipid and 8% ash (20), with the outer cyst wall being synthesized before the inner cyst wall (29). It is of interest that the outer cyst walls isolated according to the procedure presented in this paper contain nine proteins, with only one of these being a major protein band. Hence, the cyst wall protein content is very homogeneous.

The result indicating little protein homology between rat liver nucleoli and *Acanthamoeba* nucleoli is surprising since all eukaryotes synthesize ribosomes with similar sedimentation coefficients, yet the protein composition of the nucleolus, the site of assembly of these ribonucleoprotein particles (6, 7, 27), has not been preserved throughout evolution. In another study the nucleolar proteins were also observed to differ slightly between normal rat liver nucleoli, in which 97 proteins are visualized by two-dimensional gel electrophoresis, and Novikoff hepatoma ascites cell nucleoli (22), although those differences are not as striking as the ones observed in this study.

The results of the phenol-soluble nuclear protein study indicate a temporal relationship between alterations in the composition of the phenol-soluble acidic nuclear proteins and cellular differentiation during culture growth. First, there are more protein components during active exponential growth than during the stationary and poststationary phases. Second, some of the polypeptides are present only at particular phases of culture growth, either log phase or stationary and poststationary phases. Third, the relative amounts of the phenol-soluble nuclear proteins also changed. And, finally, many of these qualitative and quantitative changes began to occur specifically as cultures reached high cell density ( $2 \times 10^6$  cells/ml). It was at this stage that round forms first appeared before their encystment in late stationary phase.

In general, the results of this study are in strong agreement with recent reports of nuclear protein alterations during induced differentiation in *P. polycephalum* (14–16). Those reports have demonstrated dramatic changes in the quantitative and qualitative content of phenol-soluble nuclear proteins during induced differentiation in that amoeboid organism. *Acanthamoeba* may well represent a similar type of biological system. Of particular interest is the report (14) that amoeba actin is a major constituent of *Physarum* nuclei. Bands having a molecular weight similar or identical to that of rabbit muscle actin have been observed in the nuclear phenol-soluble fraction as well as the nucleolar phenol-soluble fraction from *Acanthamoeba*. *Acanthamoeba* actin is known to possess numerous characteristics in common with rabbit muscle actin (23, 31). Work in progress in this laboratory suggests that amoeba actin expressed as a percentage of total cellular protein increases dramatically during induced encystation (26). However, we have found that *Acanthamoeba* actin represents the most prominent band on SDS gels from whole cell homogenates and can easily contaminate organelle preparations. An analysis of the role of Amoeba actin in the nuclear events associated with induced differentiation in *Acanthamoeba* is beyond the scope of this study. In conclusion, this study has shown the isolated nucleus and nucleolus of *A. castellanii* to be a convenient and workable model system for the study of biochemical events involved in cellular differentiation.

Received for publication 25 August 1975, and in revised form 13 November 1975.

## REFERENCES

1. BALLAL, N. R., and H. BUSCH. 1973. Two-dimensional gel electrophoresis of acid-soluble nucleolar proteins of Walker 256 carcinosarcoma, regenerating liver and thioacetamide-treated liver. *Cancer Res.* **33**:2737–2743.
2. BAND, R. N. 1959. Nutritional and related biological studies on the free-living soil amoeba *Hartmannella rhyodes*. *J. Gen. Microbiol.* **21**:80–95.
3. BAND, R. N. 1963. Extrinsic requirements for encystation by the soil amoeba *Hartmannella rhyodes*. *J. Protozool.* **10**:101–106.
4. BAND, R. N., and S. MOHRLOK. 1969. The respiratory metabolism of *Acanthamoeba rhyodes* during encystation. *J. Gen. Microbiol.* **59**:351–358.
5. BULLOUGH, W. S. 1967. The evolution of differentiation. Academic Press, Inc., New York. 206.
6. BUSCH, H. 1965. Histones and other nuclear proteins. Academic Press, Inc., New York.
7. BUSCH, H., and K. SMETANA. 1970. The nucleolus. Academic Press, Inc., New York. 626.
8. CHLAPOWSKI, F. H., and R. N. BAND. 1971. Assembly of lipids into membranes in *Acanthamoeba palestinensis*. I. Observations on the specificity and stability of choline-<sup>14</sup>C and glycerol-<sup>3</sup>H as labels for membrane phospholipids. *J. Cell Biol.* **50**:625–633.
9. CHOUINARD, L. A. 1970. Localization of intranucleolar DNA in root meristematic cells of *Allium cepa*. *J. Cell Sci.* **6**:73–85.
10. CHOUINARD, L. A., and C. P. LEBLOND. 1967. Sites of protein synthesis in nucleoli of root meristematic cells of *Allium cepa* as shown by radioautography with <sup>3</sup>H arginine. *J. Cell Sci.* **2**:473–480.
11. FISHBEIN, W. N. 1972. Quantitative densitometry of 1–50 µg protein in acrylamide gel slabs with Coomassie blue. *Anal. Biochem.* **46**:388–401.
12. KNECHT, M., and H. BUSCH. 1971. Isolation of an electrophoretically homogeneous non-histone nucleolar protein. *Life Sci.* **10**:1297–1309.
13. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685.
14. LESTOURGEON, W. M., A. FORER, Y. YANG, J. S. BERTRAM, and H. P. RUSCH. 1975. Contractile proteins major components of nuclear and chromosome nonhistone proteins. *Biochem. Biophys. Acta.* **379**:529–555.
15. LESTOURGEON, W. M., and H. P. RUSCH. 1971. Nuclear acidic protein changes during differentiation in *Physarum polycephalum*. *Science (Wash. D.C.)* **174**:1233.
16. LESTOURGEON, W. M., and H. P. RUSCH. 1973. Localization of nucleolar and chromatin residual acidic protein changes during differentiation in *Physarum polycephalum*. *Arch. Biochem. Biophys.* **155**:144–158.
17. LOWRY, O. H., N. ROSEBROUGH, A. FARR, and R. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
18. MURAMATSU, M., and T. FUGISAWA. 1968. Methylation of ribosomal RNA precursor and tRNA in rat liver. *Biochim. Biophys. Acta.* **157**:476–492.
19. NEFF, R. J., S. RAY, A. BENTON, and M. WILBORN. 1964. Induction of synchronous encystment in *Acanthamoeba sp.* In *Methods in Cell Physiology*, D. M. Prescott, editor. Academic Press, Inc. New York. 55–83.
20. NEFF, R. J. and R. H. NEFF. 1969. The biochemistry of amoeba encystment. *Symp. Soc. Exp. Biol.* **23**:51–81.
21. O'FARREL, P. Z., L. M. GOLD, and W. M. HUANG. 1973. Bacteriophage T<sub>4</sub> gene expression. *J. Biol. Chem.* **248**:5499–5501.
22. ORRICK, L., M. O. J. OLSON, and H. BUSCH. 1973. Comparison of nucleolar proteins of normal rat liver and Novikoff hepatoma ascites cells by two-dimen-

- sional polyacrylamide gel electrophoresis. *Proc. Natl. Acad. Sci., U.S.A.* **70**:1316-1320.
23. POLLARD, T. D., E. SHELTON, R. R. WEIHING and E. D. KORN. 1969. Ultrastructural characterization of F-actin isolated from *Acanthamoeba castellanii* and identification of cytoplasmic filaments as F-actin by reaction with rabbit heavy meromyosin. *J. Mol. Biol.* **50**:91-97.
  24. ROVERA, G., and R. BASERGA. 1971. Early changes in the synthesis of acidic nuclear proteins in human diploid fibroblasts stimulated to synthesize DNA by changing the medium. *J. Cell Physiol.* **77**:201-211.
  25. ROVERA, G., and R. BASERGA. 1972. Early increase in nuclear acidic protein synthesis after SV40 infection. *Nat. New Biol.* **237**:240-241.
  26. RUBIN, R. W., and M. MAHER. 1975. Synthesis and degradation of actin during induced encystation in *Acanthamoeba*. *J. Cell Biol.* **67**:373 a(abstr.).
  27. SIRLIN, J. L. 1972. *Biology of RNA*. Academic Press, Inc., New York. 316-324.
  28. STEIN, G., S. CHANDURE, and B. BASERGA. 1972. Gene activation in WI-38 fibroblasts stimulated to proliferate. *J. Biol. Chem.* **247**:3918-3922.
  29. STEWART, J. R., and R. A. WEISMAN. 1974. A chemical and autoradiographic study of cellulose synthesis during the encystment of *Acanthamoeba castellanii*. *Arch. Biochem. Biophys.* **161**:488-498.
  30. WEBER, K., and M. OSBORN. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **440**:6-4412.
  31. WEIHING, R. R., and E. D. KORN. 1971. *Acanthamoeba* actin isolation and properties. *Biochemistry.* **10**:590-600.
  32. WEISENTHAL, L. M., and R. W. RUDDON. 1972. Characterization of human leukemia and Burkitt lymphoma cells by their acidic nuclear protein profiles. *Cancer Res.* **32**:1009-1017.
  33. WILHELM, J. A., A. T. ANSERVINE, A. W. JOHNSON, and L. S. HNILICA. 1972. Proteins of chromatin in genetic restriction. IV. Comparison of histone and non-histone proteins of rat liver nucleolar and extranucleolar chromatin. *Biochim. Biophys. Acta.* **272**:220-230.