THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE OCCURRENCE AND TRANSLATION OF STABLE MESSENGER RIBONUCLEOPROTEIN COMPLEXES IN THE CYTOPLASM OF *ACETABULARIA MEDITERRANEA*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

ROBERT LATURNER SHOEMAN, III

Norman, Oklahoma

1978

THE OCCURRENCE AND TRANSLATION OF

STABLE MESSENGER RIBONUCLEOPROTEIN COMPLEXES

IN THE CYTOPLASM OF *ACETABULARIA MEDITERRANEA*

APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGEMENTS

I thank Dr. Walter L. Dillard for his direction of my graduate program and his invaluable assistance in the preparation of this manuscript.

I appreciate the advice and assistance of the members of my committee: Dr. Donald Perkins, Dr. Leonard Beevers, Dr. Frank Seto and Dr. Eddie Smith.

I thank Dr. Donald Cox for serving on my committee during his tenure at the University of Oklahoma.

I thank Mrs. Mary Hagen for typing the many drafts of this dissertation.

Finally, I thank my parents, Robert L. and Elizabeth D. Shoeman, for the years of support necessary for completion of this research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

THE OCCURRENCE AND TRANSLATION OF STABLE MESSENGER RIBONUCLEOPROTEIN COMPLEXES IN THE CYTOPLASM OF ACETABULARIA MEDITERRANEA

CHAPTER I

INTRODUCTION

Acetabularia mediterranea and related species belonging to the family Dasycladaceae have been widely studied since the pioneering efforts of Hämmerling (1931, 1932, 1935, 1963). These organisms are quite large - some species reaching lengths of 200 mm (Berger, Sandakhchiev and Schweiger, 1974). They are unicellular and a single nucleus is located in the basal rhizoid during most of the life cycle. Each species proceeds through a series of characteristic stages to a characteristic adult morphology. This morphogenetic development has been described for a number of species (Berger, Sandakhchiev and Schweiger, 1974 and Schweiger, et al, 1974).

Hämmerling showed that the cells may be easily enucleated by amputating the rhizoid. The cells readily survived enucleation procedures and their morphogenetic development proceeds in a normal fashion. Enucleated fragments increase in length over a period of weeks and are capable of developing normal gametotangia (caps) and synthesizing protein in a regulated manner identical to nucleated cells several

weeks after removal of the nucleus (Spencer and Harris, 1964; Brachet, 1967; Hämmerling, 1963). Morphogenesis may be modified by transplanting nuclei from a donor species into the enucleated cytoplasm of a recipient species. Such manipulations have established that the development of the morphology that is characteristic of each species is determined by the nucleus.

These observations led Hämmerling (1932) to postulate the existence of "morphogenetische Substanzen" that were the controlling factors in morphogenesis. He postulated that these species-specific "morphogenetische Substanzen" originate in the nucleus and exhibit stability over a long period of time. The exact chemical nature of these "Substanzen" has been the subject of much research (Brachet, 1964; Hämmerling, 1963; Werz, 1969; Kloppstech and Schweiger, 1975 a,b). Messenger ribonucleic acid (mRNA) has been postulated to be identical to these "Substanzen", but final proof has been lacking.

Kloppstech and Schweiger (1975 b, 1976) have isolated polyadenylated RNA from Acetabularia mediterranea that has a number of properties that coincide with those of the "morphogenetische Substanzen": it is synthesized in the nucleus, is rapidly transported to the apical end of the cell, is stable in the cytoplasm (half-life of 10 days) and is capable of inducing peptide synthesis in a cell-free system from wheat germ (Schweiger, 1977). The nature of the peptides synthesized has not been fully investigated.

Other studies have demonstrated that the nucleus is involved in the regulation of non-morphogenetic events in the cytoplasm. For

example, the nucleus determines the differences observed in the number and electrophoretic mobility of the isoenzymes of malate dehydrogenase (Schweiger, Master and Werz, 1967; Sandakhchiev, Niemann and Schweiger, 1973; Berger, Sandakhchiev and Schweiger, 1974) and of lactate dehydrogenase (Reuter and Schweiger, 1969) among several species of Acetabularia.

The above observations are examples of the numerous lines of evidence which support the conclusion that messenger ribonucleic acid can exist for long periods of time in the cytoplasm of Acetabularia in the absence of the nucleus. This information (presumably in the form of RNA) is stable in the cytoplasm despite the existence of very active ribonucleases in intact and enucleate cells (Schweiger, 1966).

It has been postulated that the combination of mRNA with protein ("informosomes") could protect the messenger ribonucleic acid from endogenous ribonuclease, regulate the transport of the messenger ribonucleic acid from the nucleus to the cytoplasm and regulate translation by the specific binding and blocking of mRNA by regulatory proteins (Spirin, 1969). Attempts to isolate messenger ribonucleoprotein particles (mRNPs) from Acetabularia have not been successful to date. Kloppstech and Schweiger (1975a) found RNPs of buoyant density = 1.4 g/ml (in CsCl) in Acetabularia mediterranea. This is within the density range (1.35 - 1.5 g/ml in CsCl) of the informosomes in fish embryos (Spirin, 1969). However, the RNP's found by Kloppstech

and Schweiger were synthesized in enucleate as well as nucleated cells. The synthesis of these RNP's, therefore, could not be controlled by the nucleus. Alexeev, et al (1974) isolated RNPs from Acetabularia crenulata with a buoyant density of 1.4 g/ml in CsCl. These RNPs were injected into non-growing enucleate cell fragments and induced incomplete morphogenesis in 10% of the injected cells.

Messenger ribonucleic acid in the form of mRNA transcribed from nuclear genes and sequestered in the cytoplasm in the form of mRNP for long periods without translation should be detectable. It should be possible to isolate mRNP from young cells that directs the in vitro synthesis of proteins that are normally found only in older cells. We have undertaken to identify a protein unique to older cells and to demonstrate the existence in younger cells of the messenger ribonucleic acid necessary for its synthesis.

CHAPTER II

MATERIALS AND METHODS

Materials

Instant Ocean was purchased from Aquarium Systems, Inc. (Eastlake, Oh.). [5,6-^{3}H]-uridine, 40 Ci/m mole, and L-1-^{14}C-leucine, 342 mCi/m mol were purchased from Amersham Corporation (Arlington Heights, Ill.). 5-^{3}H-uridine, 26.7 Ci/m mol, L-[3,4,5-^{3}H(N)]-leucine, 78.2 Ci/m mol, L-[alanine-3-^{3}H(N)]-phenylalanine, 16.1 Ci/m mole, Aquasol liquid scintillation cocktail and PPO(2,5-diphenyloxazole) were purchased from New England Nuclear (Boston, Mass.). Tritiated radioisotopes were stored at 4°C. Carbon-14 radioisotopes were stored at -20°C. Tritiated uridine solutions were made 70% (v/v) with respect to ethanol. The ethanol was removed before use by flash evaporation.

Sephadex G-25 Medium was purchased from Pharmacia Fine Chemicals (Piscataway, N.J.). Sodium dodecyl sulfate (SDS) was recrystallized by a modification of the Decker and Foster (1966) procedure. One hundred forty grams of SDS was dissolved in 1ℓ of 70% ethanol at 70°C. One gram of clean Norit A (Thomson, 1969) was added. The mixture was filtered through Whatman #1 filter paper and precipitated at

-20°C overnight. The crystals were washed with 95% ethanol on a Buchner funnel and dried under vacuum. Acrylamide was recrystallized by dissolving 140 g in 1ℓ chloroform at 50°C and filtering through Whatman #1 filter paper. This was precipitated at -20°C overnight. The crystals were washed with cold chloroform on a Buchner funnel and dried under vacuum. N,N'-methylenebisacrylamide was recrystallized from acetone. Twenty grams were dissolved in 1ℓ, precipitated overnight at -20°C, washed with cold acetone and dried under vacuum.

Bovine serum albumin (Cohn fraction V), cytochrome C (type II-A), agarose (type 1, low EEO), and chicken egg albumin (grade V) were purchased from Sigma Chemical Company (St. Louis, Mo.). Human gamma globulin (fraction II) was purchased from Pentex (Kankakee, Ill.).

Nonidet P-40 was purchased from Deutsche Shell-Chemie GmbH (Hambürg). Cesium sulfate was purified according to Szybalski (1968) and Wilt (1973). Bentonite was purchased from Sigma Chemical Co. and purified according to Fraenkel-Conrat, Singer and Tsugita (1961).

Complete Freund's adjuvent was purchased from Difco (Detroit, Mich.). Proteinase K was obtained from E. Merck (Darmstadt). Escherichia Coli, strain D-10, was a gift from Dr. Leon Unger, University of Oklahoma Health Sciences Center. Polyribosomes were prepared from late log D-10 cultures by the method of Godson and Sinsheimer (1967). RNA was isolated from polyribosomal pellets by deproteinization with chloroform-phenol (80% w/w in water)-isoamyl alcohol (99:100:1) (Kloppstech and Schweiger, 1975b).

Xenopus laevis were purchased from Nasco (Ft. Atkinson, Wis.).

Radioactivity was determined by counting in a Beckman Model LS-133 Liquid Scintillation Spectrometer. Counts per minute were converted to disintegrations per minute (DPM) by the external standard ratio method.

Methods

Culture of Acetabularia mediterranea

Dormant cells, 1-2 mm long ("Keimlinge"), of Acetabularia mediterranea were maintained at 20°C in Müller's medium (Müller, 1962) in constant darkness. The keimlinge were illuminated at approximately 3 month intervals for 3-4 days under standard conditions of light and temperature (Keck, 1964). Growth of the cells was initiated by adding a suspension of the keimlinge stock culture containing 100-200 cells to 200 ml of sterile medium in 1 liter Roux flasks. The flasks were incubated under standard conditions. The initial cultures were subdivided when the cells reached a length of 10-15 mm, so that each Roux flask contained 50-100 cells in 200 ml of medium. All transfers or other manipulations of cells were performed under aseptic conditions.

Standard culture conditions were 21°C in Sherer RI 24 and RI 48 incubators. Lights were turned on at 08:00 CST and off at 20:00 CST. Illumination was provided by a mixture of Westinghouse "Cool-White" and General Electric "Gro-and-Sho" fluorescent bulbs. Lights were placed 12-16 cm above the culture flasks. Two thousand six hundred

ninety Lux from the "Cool-White" and 1345 Lux from the "Gro-and-Sho" bulbs illuminated the cultures. Crumpled sheets of aluminum foil were placed under the culture flasks to enhance illumination.

Müller's medium, Shephard's medium (Shephard, 1969) and modified Instant Ocean were used in various portions of the research. The compositions of the three media are given in Table 1. Müller's medium and modified Instant Ocean were sterilized by passage through a Millipore type AP 25 prefilter and a Millipore type HA filter. Two-liter volumes of Shephard's medium were autoclaved for 25 minutes at 121°C. A standard vitamin composition was adopted for use in all media. One ml of filter-sterilized standard vitamin solution was added to each liter of medium. This vitamin mixture contained 300 µg/ml thiamine-HCl, 20 µg/ml p-aminobenzoate, 10 µg/ml Ca-pantothenate, 5 µg/ml biotin, and 4 µg/ml vitamin B-12.

The bicarbonate ion concentration in modified Instant Ocean was much higher than the other media. Mature cells grown in this medium developed rigid, calcareous crusts.

Isolation and Characterization of Proteins

Acetabularia mediterranea cells were blotted dry on Whatman #1 filter paper and weighed. The cells were resuspended in a concentration of 200 mg per ml of Studier (1973) sample buffer without bromophenol blue (BPB) (50 mM Tris, pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 2 mM Na_2 EDTA and 10% glycerol) and homogenized in a Potter-Elvejehm homogenizer on an ice bath. The homogenate was centrifuged at 10,000 xg

TABLE 1. Synthetic media for Acetabularia mediterranea. Concentration of all solutes given in mM.

	Müller's	Shephard's	Modified Instant Ocean[1]
NaCl	475	410	441*
$MgSO_4$	26.6	48.7	26*
$MgCl_2$	23		23.5
$CaCl_2$	13.5	6.8	9.3*
KCl	9.8	10	9.5*
Tris[2]		8.3	
$NaHCO_3$	2.4	1.2	7.67*
$NaNO_3$	1.18	0.47	0.472
H_3BO_3	0.032	0.03	0.4
NaBr	0.185		0.27
SiO_2			0.2*
Na_2HPO_4	0.14		0.01
$SrCl_2$	0.014		0.09
Na_2SiO_3	0.07		
Na_2 EDTA[3]	0.054	0.032	0.00016
LiCl	0.00014		0.029
$MnCl_2$		0.001	0.018
$ZnCl_2$	0.014		0.0003
$ZnSO_4$		0.007	
NaF			0.005*
K_2HPO_4		0.006	
$MnSO_4$	0.0038		
Na_2MoO_4	0.0008	0.004	0.004
S_2O_3			0.0036
$FeCl_3$		0.002	0.0005*
$AlCl_3$	0.00021		0.0015
Fe(2) Citrate	0.0012		
RbCl	0.00013		0.001
NaI			0.0006
VCl_2			0.0004
$CoCl_2$	0.00004	0.00001	0.00017
KI	0.00012		
$CuSO_4$	0.000008	0.00001	0.00005

[1] $NaNO_3$ (0.47 m moles/l) was added to Instant Ocean salts that had been dissolved in University of Oklahoma tap water. The salt concentrations marked with an asterisk (*) are the sum of those in the Instant Ocean preparation and those in the tap water. The tap water analysis is that reported in Clemens and Jones (1954).

[2] Tris is 2-amino-2-(hydroxymethyl)-1,3-propanediol.

[3] Na_2 EDTA is (Ethylenedinitrilo)tetraacetic acid, disodium salt.

for 10 min. at 0°C. Low molecular weight materials which interfered with electrophoretic separation were removed from the supernatent by gel filtration. Small sample volumes (1-3 ml) were applied to a 15 x 60 mm column of Sephadex G-25 Medium that had been equilibrated with Studier buffer without BPB. Larger samples (3-8 ml) were applied to a 15 x 150 mm column. The effluent of the column was monitored at 280 nm with an Isco UA-5 Absorbance Monitor and Type 6 Optical Unit (Instrumentation Specialty Co., Lincoln, Neb.). The peak that eluted in the void volume was collected. Trichloracetic acid (TCA) was added to a final concentration of 10% (w/w) and the sample was left overnight at 4°C. The precipitate was collected by centrifugation at 10,000 xg for 10 min. at 0°C. The precipitate was washed 4 times with 95% ethanol, twice with acetone and twice with diethyl ether. The ether was removed under vacuum. The proteins were then stored at -70°C.

Xenopus laevis oocytes were homogenized by suspending 25 oocytes in 1 ml of Studier buffer without BPB and forcing the suspension through a 1" 23 ga needle. The homogenate was then centrifuged at 13,000 rpm in a Beckman Model 152 Microfuge. The supernatent was carefully pipetted off, avoiding the yolk platelets at the meniscus, and made 10% (w/w) with respect to TCA. The remainder of the procedure was identical to that for Acetabularia mediterranea.

Polyacrylamide gel electrophoresis separations were made on 10 x 13 cm slabs using the apparatus designed by Studier (1973). Proteins were separated with the continuous buffer system (25 mM Tris, 192 mM glycine, pH 8.3, 2 mM Na_2 EDTA, 0.1% SDS) of Laemmli (1970). Gels

of 7.5%, 10% and 12% acrylamide were prepared with 3% acrylamide stacking gels. Analytical separations were made on 1.1 mm thick gels. Preparative separations were made on 3 mm thick gels.

TCA-precipitated proteins were dissolved in Studier sample buffer, placed in Beckman microfuge tubes and heated in a boiling water bath for 2-5 minutes. The samples were centrifuged at 13,000 rpm for 2 minutes. A mixture of bovine serum albumin (300 µg/ml), human gamma globulin (1 mg/ml), chicken egg albumin (1 mg/ml) and cytochrome C (400 µg/ml) was used as a molecular weight standard. An appropriate volume (10-30 µl) of this mixture was run on every gel.

Electrophoresis was carried out at 30-40 mA. The run was usually terminated when the BPB band reached the lower edge of the gel. Protein bands were stained in Coomassie Brilliant Blue R250 at room temperature according to Maizel (1971) and destained electrophoretically. Radioactive proteins were detected by fluorography (Bonner and Laskey, 1974; Laskey and Mills, 1975). Stained gels and fluorography films were scanned with an Isco model 1310 gel scanner.

Radioisotope Labeling of Macromolecules

Freshly-prepared Müller's medium was found to provide the highest rate of incorporation of labeled precursors into macromolecules. One-half gram of keimlinge was placed in 20 ml of incorporation medium contained in 100 ml milk dilution flasks. The incorporation medium contained 5-10 µCi/ml of ^{3}H-labeled precursors and/or 1-2 µCi/ml of ^{14}C-labeled precursors. Older cells were treated similarly except

that they were incubated in 10 ml of incorporation medium. Incorporation was terminated by washing the cells 3 times with a total of 100 ml of freshly-prepared Müller's medium containing a 30-100 fold excess (0.2 mg/ml) of unlabeled precursor.

Isolation of Messenger Ribonucleoproteins and Messenger Ribonucleic Acids

Acetabularia mediterranea keimlinge and cells 5 mm long were labeled for 28 hours, starting 2 hours after the beginning of the light period, in the presence of ^{3}H-uridine and ^{14}C-leucine. Messenger ribonucleoproteins were isolated using modifications of Greenberg's (1977) technique. Cells were resuspended at a concentration of 0.3 g per ml in sample buffer A (50 mM Tris, pH 7.5, 30 mM KCl, 2 mM $MgSO_4$, 2 mM 2-mercaptoethanol, 0.5% Nonidet P-40, 0.1% betonite and 10% (v/v) glycerol) and homogenized in a Potter-Elvejehm homogenizer on an ice bath. The homogenate remained on ice for 5 min. and was then centrifuged at 10,000 xg for 10 min. at 0°C in polycarbonate tubes. The supernatent volume was adjusted to 3.54 ml by the addition of Cs_2SO_4 gradient buffer (50 mM Tris, pH 7.5, 30 mM KCl, 2 mM $MgSO_4$, 0.01% Nonidet P-40 and 15% (v/v) dimethyl sulfoxide). The diluted supernatent was brought to a final volume of 5.9 ml by the addition of 1.69 molal Cs_2SO_4 dissolved in gradient buffer. This solution was then layered over 5.9 ml of 1.69 molal Cs_2SO_4 in 13.5 ml polyallomer tubes. The tubes were centrifuged for 30 hours at 46,000 rpm at 25°C in the Beckman Type 65 rotor. The bottom of the tubes was punctured and the

gradients were displaced by injection of 0.4 ml volumes of mineral oil delivered with a Hamilton Repeating Dispenser (Hamilton Co., Reno, Nevada). Five or 10 µl of each fraction and 50 µl of 1 N HCl were added to 5 ml Aquasol. The density of each fraction was determined by weighing 100 µl aliquots on an analytical balance. The gradient fractions were stored at -70°C while the liquid scintillation counting was performed.

Gradient fractions containing mRNP were pooled and diluted 50-fold with 10 mM Tris buffer, pH 7.5 containing 500 mM KCl and 1 mM $MgCl_2$. The mRNP was separated into 2 fractions by filtering through nitrocellulose filters (Greenberg, 1977). The nitrocellulose-filter-bound RNP was eluted from the filter by shaking for 30 min. with 2 changes of 5 ml of 0.5% SDS and 200 µg/ml proteinase K. The RNP was deproteinized with chloroform-phenol-isoamyl alcohol. The RNA was precipitated for 2 hrs. after the addition of 650 µg of E. coli ribosomal RNA by the addition of 2 volumes of 95% ethanol containing 0.2 M sodium acetate at -20°C. The precipitate was collected by centrifugation at 16,000 xg for 10 min. at 0°C. The RNA pellet was drained and stored at -70°C.

One-tenth volume of 1% SDS and 200 µg/ml proteinase K was added to the filtrate solution. This was then concentrated 40-fold by dialysis against saturated polyethylene glycol (molecular weight range: 6000-7500) dissolved in 10 mM Tris buffer, pH 7.5, containing 500 mM NaCl and 1 mM $MgCl_2$. The inside of the dialysis bag was washed with 4 volumes of glass distilled water and the washings added to the concentrated

RNP solution. This preparation was then deproteinized and precipitated with ethanol.

Microinjection of Xenopus Oocytes

Each experiment utilized oocytes from the same Xenopus laevis female. Oocytes were injected with 50-80 nl of mRNA dissolved in Barth's medium (Barth and Barth, 1959 and Gurdon, 1968). Each mRNA sample was injected into 10 oocytes (Gurdon, Lane, Woodland and Marbaix, 1971). Control oocytes were injected with Barth's medium or E. coli rRNA. The oocytes were incubated for 24 hrs. at 21°C in 2 ml disposable beakers covered with coverslips and sealed with silicone grease. Each beaker contained 100 µl of Barth's medium containing 100 µCi/ml of ^{3}H-phenylalanine neutralized with 0.1 N NaOH.

Determination of Site of Synthesis of mRNP

Enucleated fragments from cells 20-25 mm long were prepared by ligating and amputating the rhizoid and 5 mm of the apical end. Nucleated control cells had only the apical 5 mm amputated. The cells were allowed to recover overnight. Both types of cells were incubated for 28 hrs. in the presence of 5 µCi/ml ^{3}H-uridine and 40 µg/ml rifampin beginning one hour after the onset of the light phase. The cells were homogenized and subjected to Cs_2SO_4 isopycnic density gradient centrifugation as described.

Preparation and Use of Immunoadsorbant

The MY protein from 3.8 g of mature (30-35 mm) Acetabularia

mediterranea cells was isolated by preparative polyacrylamide gel electrophoresis on 7.5% gels, followed by separation on 12% gels. The protein was recovered from the 7.5% gels by homogenization in 10 volumes of Studier buffer (containing one-tenth the normal concentration of SDS and without BPB). The gel fragments were removed by centrifugation at 16,000 xg for 10 min. at 0°C. The supernatent was then lyophilized. The same procedure was used to recover the protein from the 12% gels, except 20 mM Tris buffer, pH 7.4 was used.

One hundred thirty milligrams of lyophilized MY protein was isolated and resuspended in 3.2 ml of 0.15 M NaCl. This was divided into 0.8 ml aliquots and stored at -70°C. Just before injection, 0.8 ml of freshly-thawed protein solution was mixed in a Potter-Elvejehm homogenizer with 0.8 ml 0.15 M NaCl and 1.6 ml of complete Freund's adjuvant.

One ml of this mixture was injected into each of 3 domestic rabbits (Oryctolagus sp.). The first injection was made subcutaneously dorsal to the pectoral girdle. The second injection was given subcutaneously dorsal to the pelvic girdle 6 days later. Three days after the last injection, 1.5 ml of blood was removed from each rabbit and tested for the presence of antibody using an interfacial precipitation test (Kwapinski, 1972). Ten days after the first injection, 15 ml of blood was removed by cardiac puncture from rabbits that showed a positive reaction for antibody. Fifteen ml of blood was also removed from an uninjected control rabbit.

Insoluble immunoadsorbants were prepared from the rabbit antisera (Avrameas and Ternynck, 1969). Tritium-labeled proteins (20 µl or 50 µl) produced in Xenopus oocytes injected with Acetabularia mediterranea mRNA were mixed with immunoadsorbant (14 mg or 70 mg) in 1.5 ml microfuge tubes. The mixture was shaken for 30 min. at room temperature and centrifuged for 2 min. in the Beckman microfuge. The supernatent was decanted and saved. The pellet was washed twice with a volume of buffered saline (150 mM NaCl in 10 mM Tris buffer, pH 7.4) equal to the original volume. Unbound radioactivity was determined by combining all supernatents and counting in 10 ml of Aquasol.

Specifically-bound antigen was eluted from the immunoadsorbant by washing three times with one volume of 100 mM glycine buffer, pH 2.8. The eluate was counted in 10 ml of Aquasol.

CHAPTER III

RESULTS

Growth of *Acetabularia mediterranea*

Cells grew most rapidly in Müller's medium. Cells attained a length of 5 mm in 10-14 days, a length of 15 mm in 30-35 days, and a length of 30 mm in 60-70 days. Gametotangia (mature caps) were well formed by 70-80 days and were filled with gamete-bearing cysts after 80-90 days.

Isolation and Characterization of Proteins

Figure 1 shows a typical calibration curve for molecular weight estimation. SDS-polyacrylamide gel electrophoresis was carried out on 10% gels for this figure. Figure 2 shows the proteins from *Acetabularia mediterranea* cells of 15 mm and 30 mm in length. These proteins were separated on 10% SDS-polyacrylamide gels and stained with Coomassie Blue. Figure 3 shows the proteins from cells of 5 mm, 15 mm and 30 mm in length that were labeled with ^{3}H-leucine. These proteins were separated on 10% SDS-polyacrylamide gels and fluorographed.

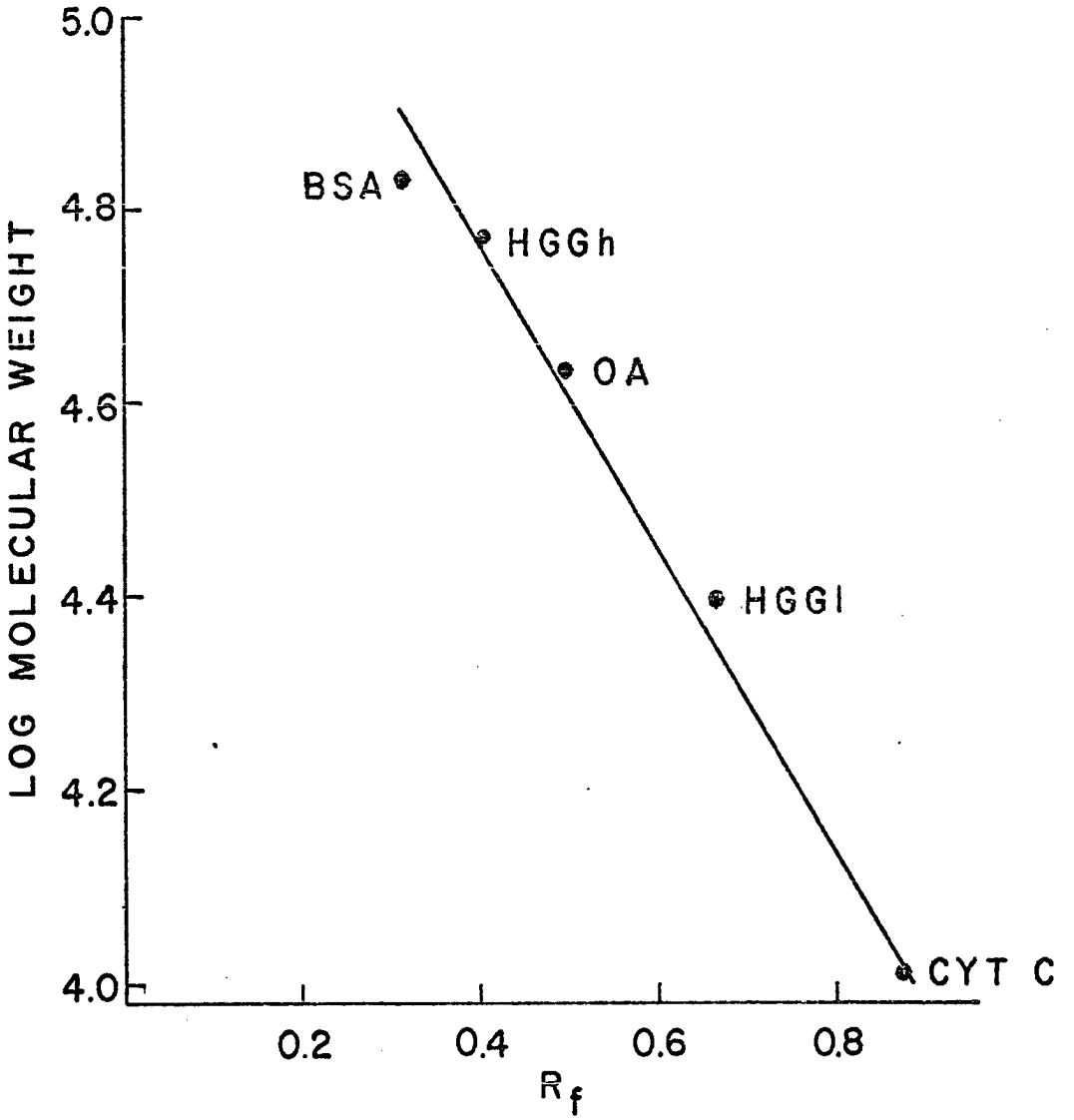

Figure 1

Calibration curve for molecular weight estimation. Molecular weight standards are bovine serum albumin (BSA) = 68,000; human gamma globulin heavy chain (HGGh) = 55,000; chicken egg albumin (OA) = 43,000; human gamma globulin light chain (HGGl) = 25,000; and cytochrome C (CYT C) = 12,000. Equation of best fit line is: log MW = -1.351 (Rf) + 5.279; (r = 0.997).

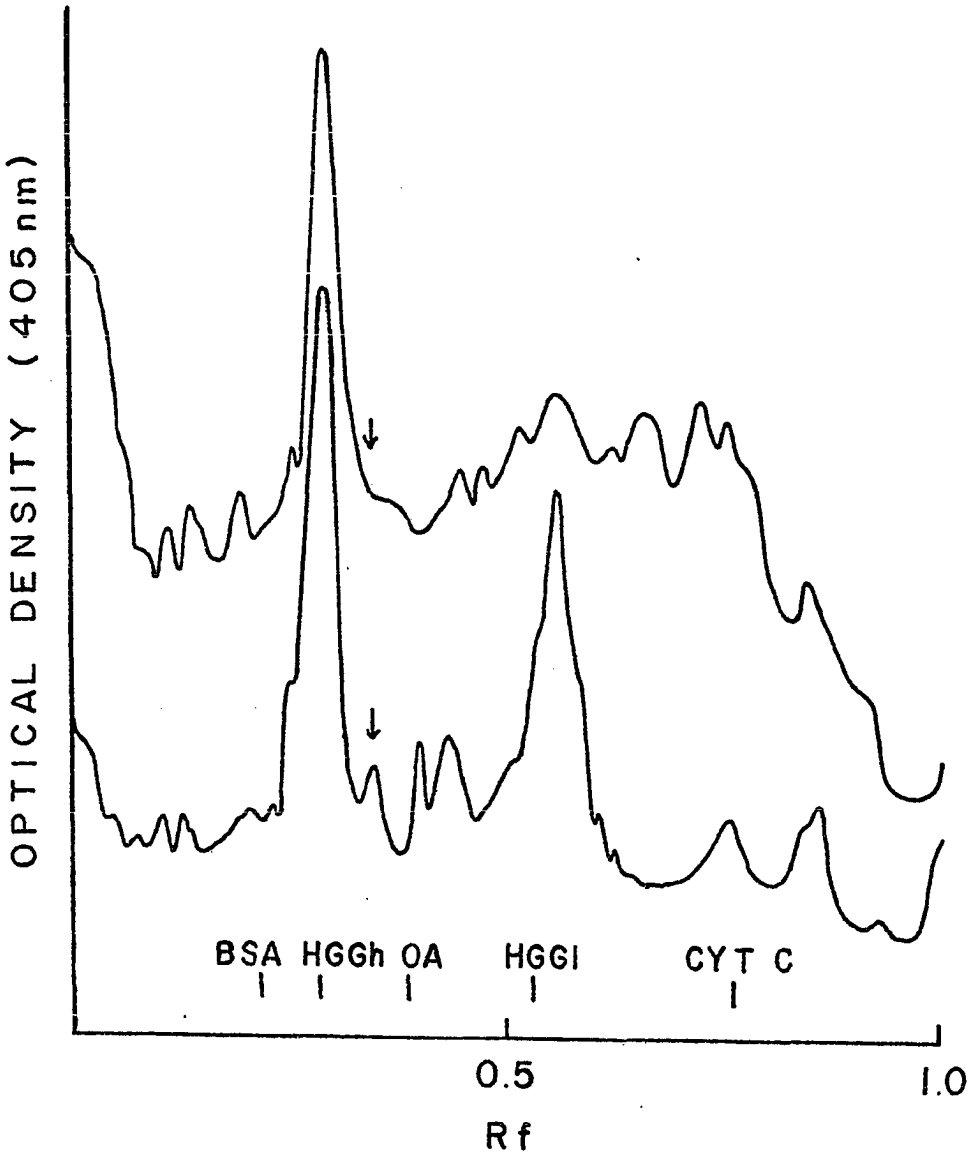

Figure 2

Scan of Coomassie Blue-stained proteins from Acetabularia mediterranea. Upper curve: protein from cells 15 mm long. Lower curve: protein from mature cells (30 mm long). The arrow indicates the position of the MY protein.

Figures 2 and 3 show the presence of a unique protein, designated MY, in the older cells. No trace of this protein was found in 5 mm cells. There was a low rate of synthesis and a barely detectable concentration of the MY protein in 15 mm cells. The MY protein has a molecular weight of 51,000.

Figure 7 shows the proteins from injected Xenopus laevis oocytes that were labeled with ^{3}H-phenylalanine and fluorographed. The high endogenous protein synthesis of the oocytes is evident from the control injected oocytes. There is an endogenous Xenopus protein which migrates coincident with the MY protein in electrophoresis.

Isolation of Messenger Ribonucleoproteins and Messenger Ribonucleic Acids

The RNPs isolated from 20-25 mm long enucleated cells and nucleated control cells are shown in Figure 4. Incorporation of ^{3}H-uridine into ribosomal RNA was inhibited by the use of rifampin at 20 µg/ml. A large peak of activity (density = 1.33 g/ml) was found in the nucleated control cells. Greenberg (1977) found mRNP from mouse L-cells to have a density of 1.3-1.5 g/ml under similar conditions.

The RNPs isolated from keimlinge and 5 mm cells are shown in Figures 5 and 6, respectively. RNP was labeled with ^{3}H-uridine and ^{14}C-leucine. The Cs_2SO_4 density gradient centrifugation strips proteins from ribosomal RNA. mRNP particles remain intact under these conditions. Radioactive label incorporated into ribosomal proteins was found near the meniscus after centrifugation (density = 1.1-1.2 g/ml) and label

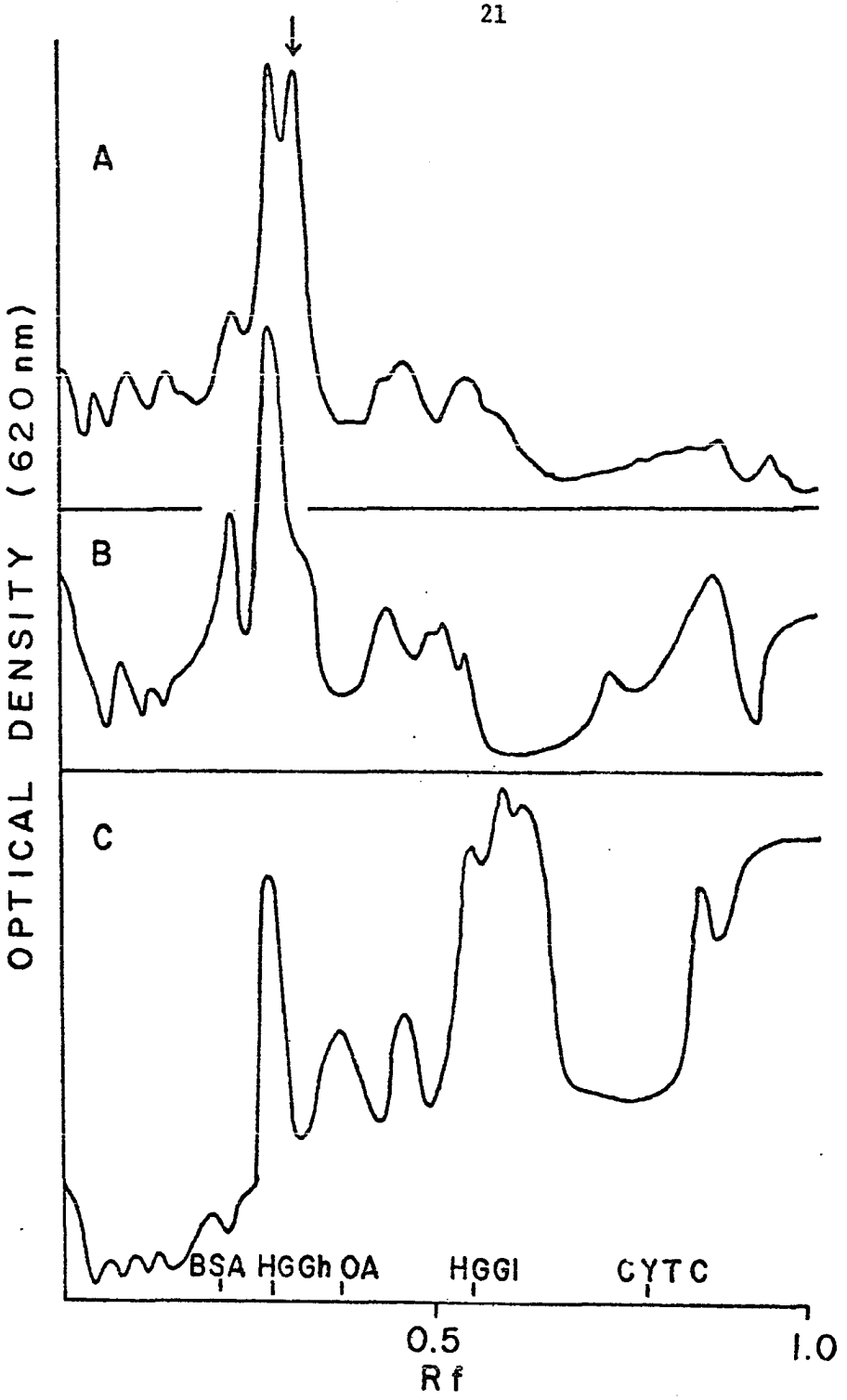

Figure 3

Scans of fluorographs of ^{3}H-labeled proteins from Acetabularia mediterranea. A = mature cells (30 mm long). B = 15 mm long cells. C = 5 mm long cells. The arrow indicates the position of the MY protein.

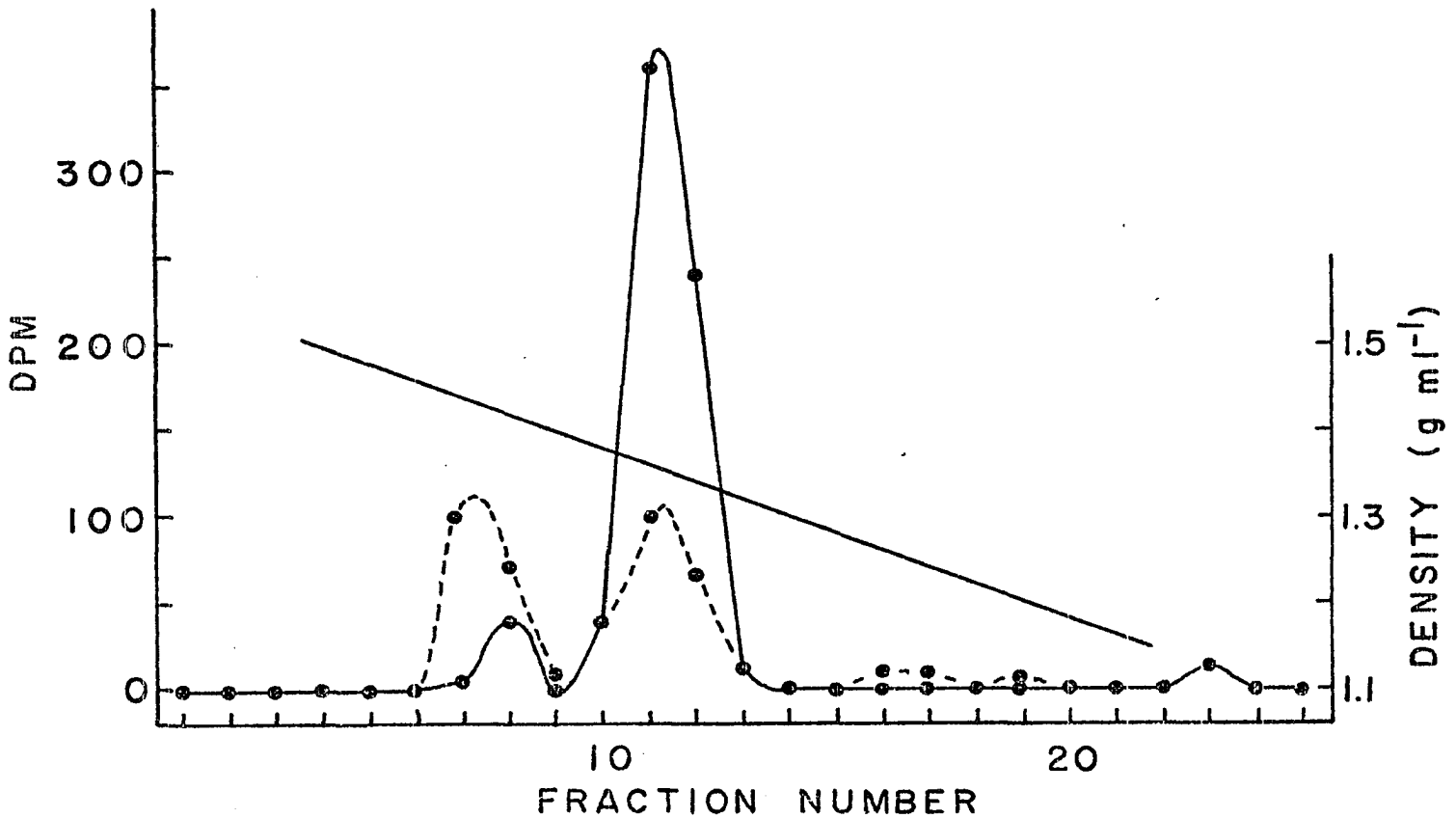

Figure 4

Isopycnic Cs_2SO_4 density gradient centrifugation of ^3H-labeled RNP from intact cells (—●—●—) and enucleate cells (- - ● -●-) of Acetabularia mediterranea. Cells 20-25 mm long were labeled for 24 hrs. with ^3H-uridine in the presence of 20 µg/ml rifampin.

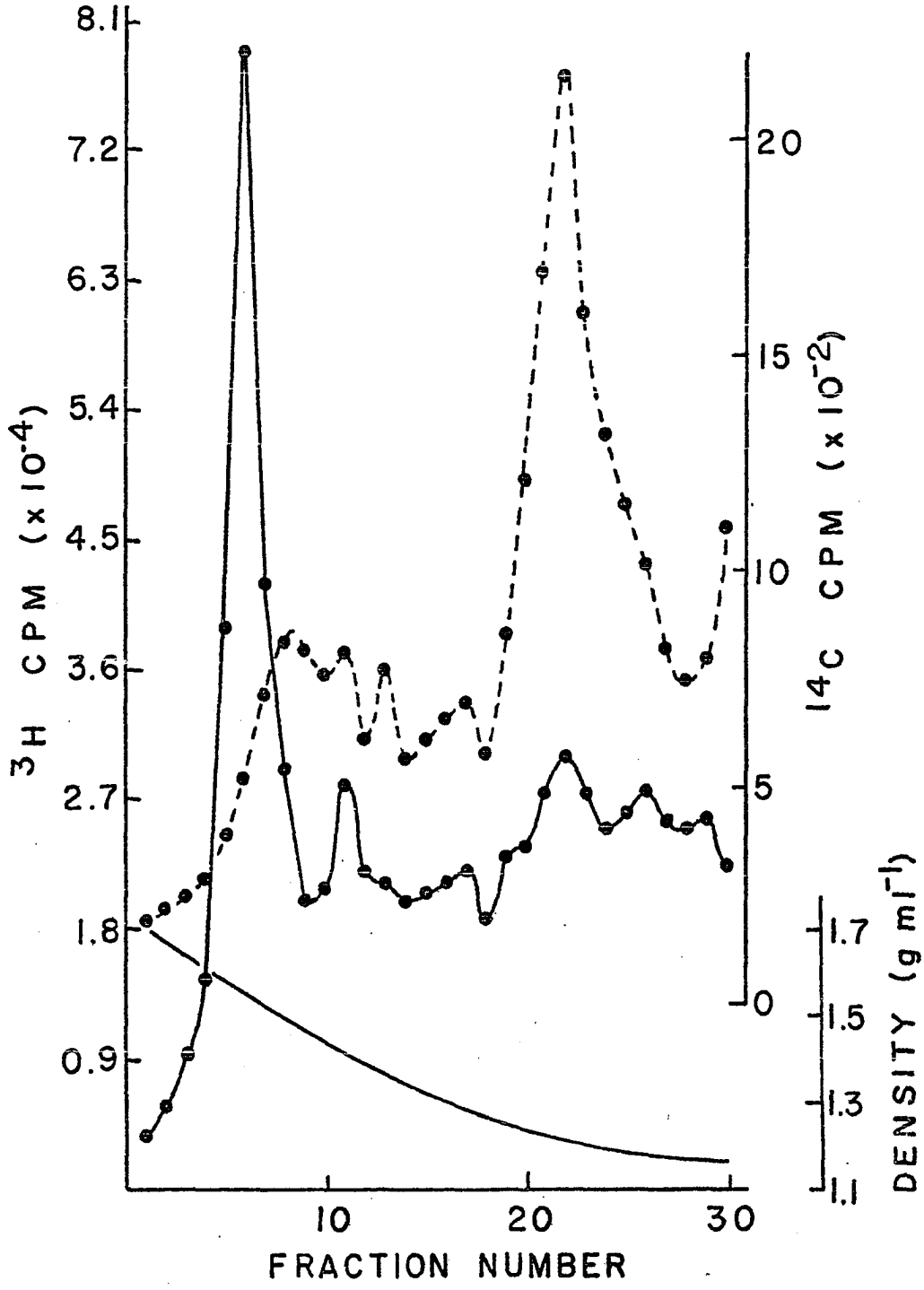

Figure 5

Isopycnic Cs_2SO_4 density gradient centrifugation of RNP from keimlinge. Protein is labeled with ^{14}C (-●-●-). RNA is labeled with ^{3}H (—⊖—⊖—).

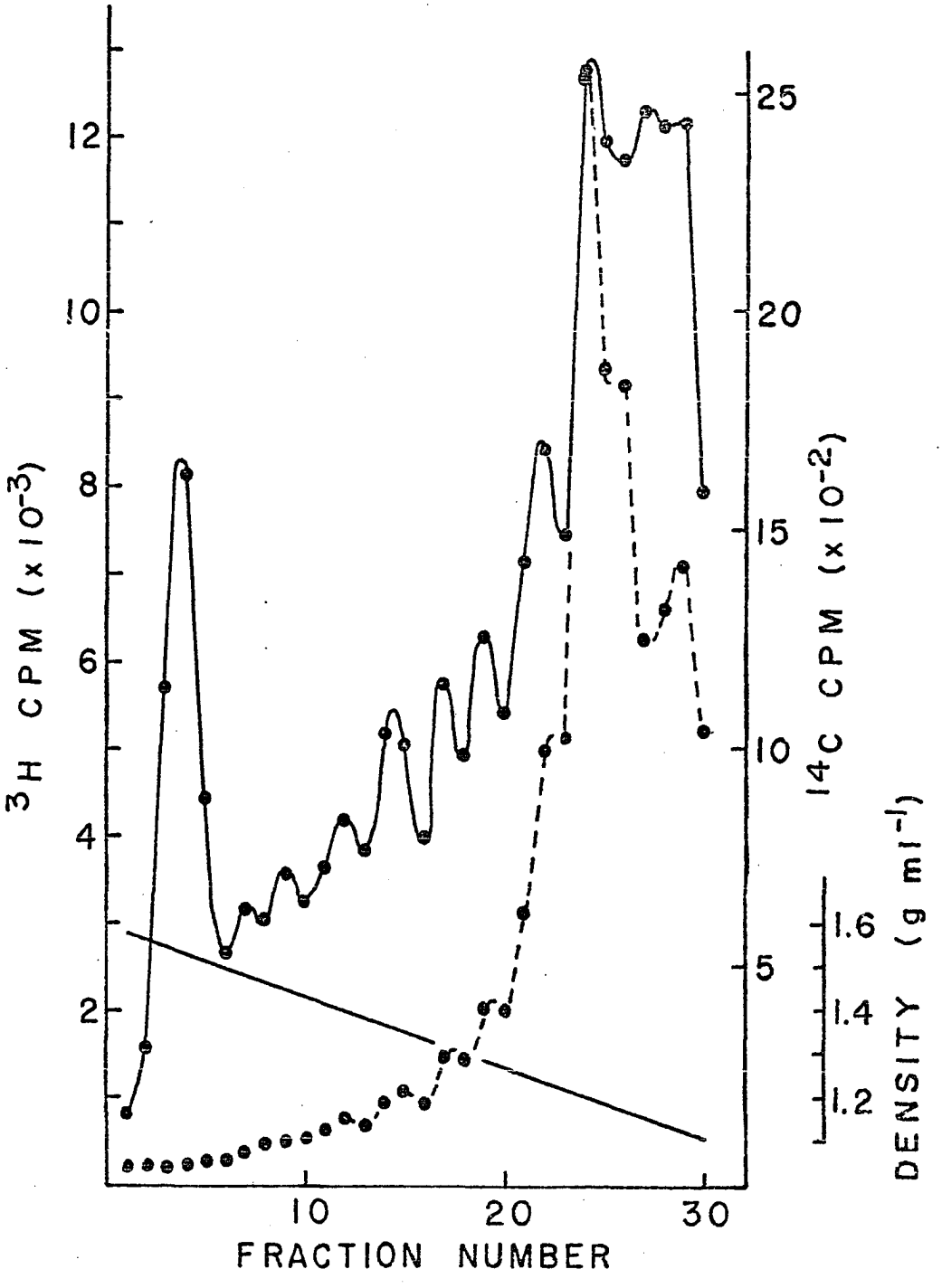

Figure 6

Isopycnic Cs_2SO_4 density gradient centrifugation of RNP from 5 mm cells. Protein is labeled with ^{14}C (—●—●—). RNA is labeled with ^3H (—●—●—).

incorporated into ribosomal RNA is found near the position of pure RNA (1.57 g/ml). mRNP particles are found in the density range of 1.3-1.5 g/ml (Greenberg, 1977). Figures 5 and 6 both show peaks of activity corresponding to protein, mRNP and ribosomal RNA. Figures 5 and 6 differ in the heterogeneity of the mRNP fraction.

Fractions 10-18 of Figure 5 were pooled. Fractions 6-22 of Figure 6 were also pooled. The RNP was fractionated by binding to a nitrocellulose filter. RNP that bound to the nitrocellulose filter was designated filter (+). RNP that did not bind to the filter was designated filter (-). The RNA from both types of RNP was isolated by deproteinization.

Protein Synthesis in *Xenopus* Oocytes

Xenopus oocytes were injected with filter (+) RNA, filter (-) RNA and *E. coli* rRNA or Barth's medium as a control. The *Acetabularia mediterranea* RNA injected in this experiment was obtained from cells 5 mm long. Protein with a molecular weight equal to the MY protein is apparent in the injected oocytes. However, in the control there is a low level of incorporation of radioactivity into an endogenous *Xenopus* protein of the same molecular weight.

Preparation and Use of Immunoadsorbant

Three rabbits were injected with purified MY protein. Two rabbits developed anti-MY antibodies, as indicated by an interfacial precipitation test. Blood was removed again the following day in

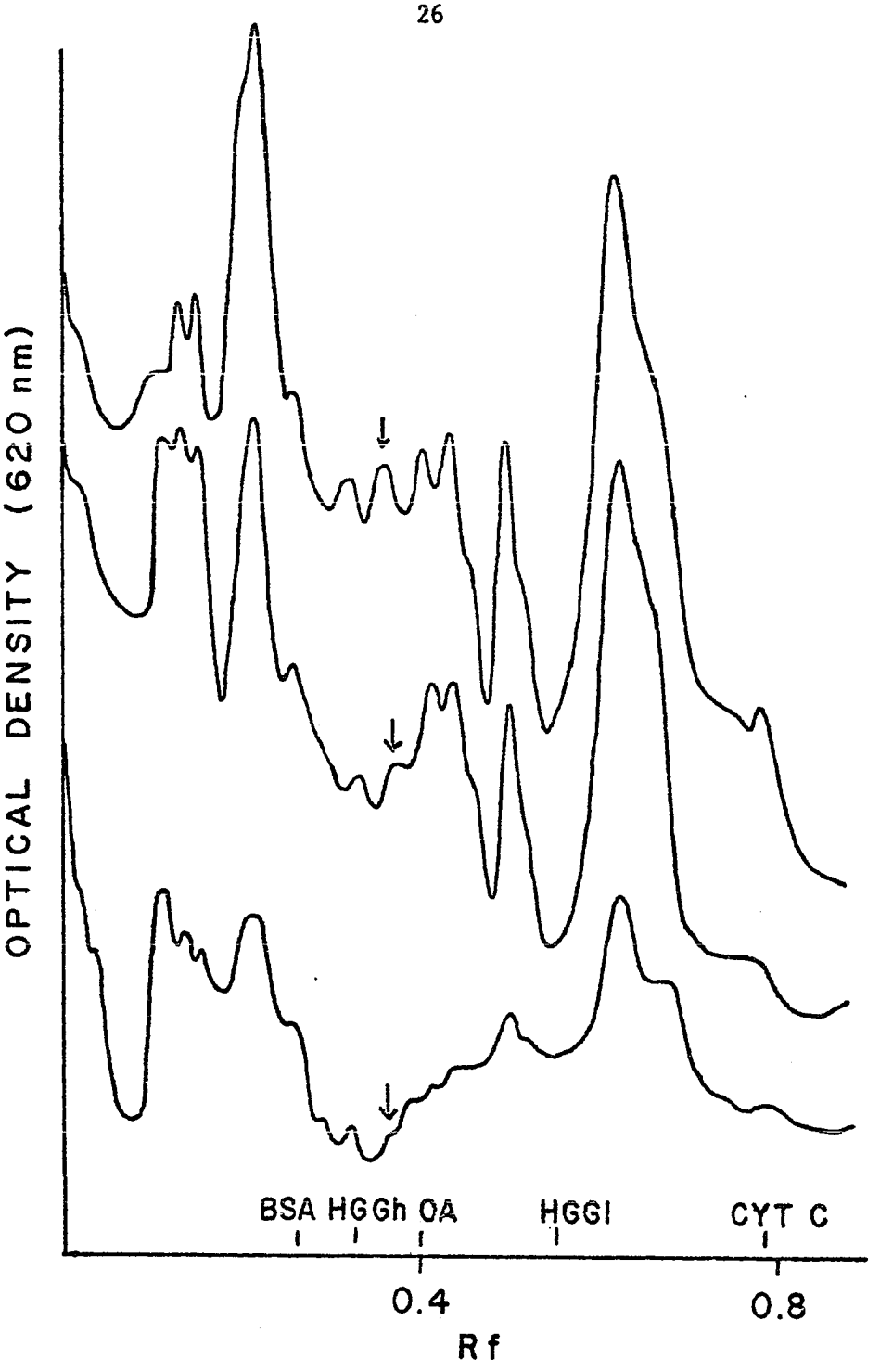

Figure 7

Scan of fluorograph of ^{3}H-labeled proteins from injected Xenopus laevis oocytes. Upper curve: injected with filter (+) RNA. Middle curve: injected with filter (-) RNA. Lower curve: injected with Barth's medium (control). The arrow indicates the position of proteins with MW = 51,000.

large quantity and again tested for anti-MY antibody. One rabbit's serum was found to contain a satisfactory titer of anti-MY antibody. Blood was removed from an uninjected rabbit for use as a control serum.

Immunoadsorbants made from each serum were mixed with Xenopus oocyte proteins produced after injection of filter (+) RNA, filter (-) RNA and Barth's medium. The RNA from the keimlinge RNP was injected into Xenopus oocytes without fractionation into filter (+) and filter (-). The proteins produced were mixed with both types of immunoadsorbant. Table 2 shows the results of all the immunoadsorbant binding experiments.

Two percent or less of the input radioactivity of proteins isolated from oocytes that had been injected with Barth's medium or keimlinge RNA was bound to the anti-MY antiserum. Proteins isolated from filter (+) - and filter (-) - injected oocytes bound specifically to the anti-MY antiserum at levels exceeding 10% of the input radioactivity.

The affinity of the proteins translated from filter (+) RNA was 55 times greater for the anti-MY antiserum than for the control antiserum. Its affinity for anti-MY antiserum was 10 times greater than that of proteins from control oocytes. The average affinity of the proteins translated from filter (-) RNA for the anti-MY antiserum was 6.1 times greater than for the control serum. Its affinity for anti-MY antiserum was 13.4 times greater than that of protein from control oocytes. Proteins translated from keimlinge RNA showed no

TABLE 2. Binding of MY protein to immunoadsorbant.

Immuno-adsorbant	Oocytes						
	Barth's injected		Filter (+) injected	Filter (-) injected		Keimlinge RNA injected	
	Run 1	Run 2		Run 1	Run 2	Run 1	Run 2
	Input radioactivity (DPM)						
	965	1040	876	1068	1075	1474	1272
	Unbound radioactivity (DPM)						
anti-MY	430	602	460	420	732	355	487
control	395	594	370	353	639	337	524
	Specifically-bound radioactivity (DPM)						
anti-MY	3	19	96	183	132	12	25
control	15	5	2	23	30	12	24
	Percent of input bound						
anti-MY	0.3	1.8	11.0	17.1	12.3	0.8	2.0
control	1.6	0.5	0.2	2.2	2.8	0.8	1.9

Filter (+) was the RNA from the RNP that bound to a nitrocellulose filter.

Filter (-) was the RNA from the RNP that did not bind to a nitrocellulose filter.

specific affinity for the anti-MY antiserum (ratio of radioactivity bound to anti-MY antiserum to radioactivity bound to control = 1.0-1.1).

A significant fraction of the input radioactivity was not recovered in the combined radioactivity of the unbound material and the bound and eluted radioactivity. This fraction probably represents non-specifically bound radioactivity and specifically bound radioactivity that was not eluted (Avrameas and Ternynck, 1969).

CHAPTER IV

DISCUSSION

The MY protein has the properties necessary to test the hypothesis of long-term storage of genetic information in the cytoplasm. It is undetectable in cells 5 mm long or younger. It occurs in the highest concentration and is synthesized at the highest rates in older cells. The linear response of pre-exposed x-ray films in the fluorographic detection of radioactivity provides a means of accurately measuring the rate of synthesis (Laskey and Mills, 1975).

Ribonucleoprotein particles capable of directing the synthesis of the MY protein are found in young cells which do not synthesize the MY protein. No significant incorporation of ^{3}H-uridine into RNP was found in enucleate cells. Nucleated cells, on the other hand, had high levels of incorporation of ^{3}H-uridine into RNP with a buoyant density of 1.3 g/ml. The occurrence of MY protein-specific ribonucleoprotein particles is therefore nucleus dependent.

Isopycnic Cs_2SO_4 density gradient centrifugation dissociates ribosomes into ribosomal core particles (essentially bare rRNA, buoyant density = 1.57 g/ml) and free ribosomal proteins (buoyant density = 1.1-1.2 g/ml). Other RNPs, presumably messenger RNPs, are more resistant

to dissociation by high Cs_2SO_4 concentrations and are found in the density range of 1.3 - 1.5 g/ml (Greenberg, 1977). In gradients containing homogenates from keimlinge and 5 mm long cells, peaks of radioactivity corresponding to ribosomal core particles and ribosomal proteins were found. Both ^3H and ^{14}C radioactivity were distributed in a series of peaks in the density range of 1.28 - 1.5 g/ml. Thus these peaks contain both RNA and protein. They must represent a stable association of RNA and protein (viz, RNP) since the buoyant density of free RNA and free protein is significantly different from the observed density of 1.28 - 1.5 g/ml (Greenberg, 1977).

The distribution of radioactivity in this region of the gradients was heterogeneous and quite different in cells of different ages. Heterogeneity of messenger-containing RNPs would be expected - corresponding to the diversity of proteins that are synthesized by the cell. It is also reasonable to expect a difference in the mRNPs found in cells of different ages. The types and numbers of proteins required is presumably different during different stages of development.

The MY protein was synthesized in oocytes injected with filter (+) RNA and filter (-) RNA from the 5 mm long cells. There was a low level of synthesis of an endogenous Xenopus protein of MW = 51,000. Thus the unequivocal identification of the MY protein by SDS-PAGE was not possible.

The occurrence of MY protein messenger activity in both the filter (+) RNA and filter (-) RNA was surprising. It was expected that

all the messenger activity would be found in RNP bound to the nitrocellulose filter (Greenberg, 1977). It seems unlikely that there exist two functionally different mRNPs for the same protein. A plausible explanation for MY protein messenger activity in both filter (+) RNA and filter (-) RNA is that the nitrocellulose filter became saturated with RNP and the additional RNP was unable to bind and thus would be found in the filtrate solution. Or, perhaps the specific affinity of nitrocellulose filters for RNP is not absolute (Infante and Nemer, 1968).

Proteins isolated from homogenates of Xenopus oocytes injected with filter (+) RNA and filter (-) RNA from 5 mm long cells bind specifically to immobilized rabbit anti-MY antibody. These results unequivocally establish the presence of the MY protein in these homogenates. No MY protein was synthesized in oocytes injected with keimlinge RNA.

A significant fraction of the input radioactivity was irreversibly bound to both immunoadsorbants. Avrameas and Ternynck (1969) have noted that immunoadsorbants made from unfractionated antiserum have a high level of non-specific binding. These observations were made in procedures which used purified antigen preparations. The antigen preparations which contained the MY protein were derived from whole cell homogenates of Xenopus oocytes and were thus extremely complex mixtures of many proteins. The quantity of MY protein in the homogenates is very small (a maximum estimate is 17 ng/ml, assuming the

MY protein contains 5% phenylalanine and all the phenylalanine was ^{3}H-labeled at 100% isotopic enrichment). Therefore, the irreversibly bound radioactivity can be attributed to protein other than the MY protein.

The results presented here demonstrate that genetic regulation in Acetabularia may occur at the level of translation. Models of regulation of protein synthesis that deal with control at the transcriptional level, such as the Jacob and Monod (1961) "operator model", can have no relevance to enucleate cells. The unique characteristics of Acetabularia make it an ideal model system for investigating alternative mechanisms of regulation. Spencer and Harris (1964) found that the activity of an alkaline phosphatase enzyme (pH 12 enzyme) was increased just prior to cap formation in enucleate as well as intact cells. They concluded that "the cytoplasm of Acetabularia contains independent mechanisms... for the continuous regulation of synthesis over a period of weeks". They suggested that such mechanisms may represent a general phenomena. These results give some indication of the possible nature of these independent cytoplasmic mechanisms. Although regulation at the transcriptional level has been unequivocally demonstrated in eucaryotes (Rowe and Wyngaarden, 1966 and Martin and Owen, 1972), it is clearly not the only means and probably not the principle mechanism of regulation in these cells.

SUMMARY

Proteins from Acetabularia mediterranea cells 5 mm, 15 mm and 30 mm long were separated by SDS-PAGE. A protein, designated MY, of MW = 51,000 was found to occur only in older cells. This protein was isolated and purified by preparative SDS-PAGE. An anti-MY antibody was produced in rabbits.

RNPs were isolated from keimlinge and 5 mm long cells. The RNPs were of density = 1.25 - 1.5 g/ml in Cs_2SO_4. RNA was isolated from these RNPs and injected into Xenopus laevis oocytes. MY protein produced by translation of the Acetabularia mediterranea mRNA in Xenopus oocytes was detected by SDS-PAGE and reaction with an anti-MY immunoadsorbant. Keimlinge mRNA did not direct the synthesis of MY protein. mRNA from 5 mm long cells was effective in directing the synthesis of MY protein.

These results demonstrate the long-term cytoplasmic storage of genetic information in Acetabularia and suggest that genetic regulation in Acetabularia may occur at the level of translation.

LITERATURE CITED

Alexeev, A. B., M. I. Betina, S. L. Stvolinsky, A. A. Yazykov and T. N. Zubarev. 1974. Evidence for a relationship between some ribonucleoprotein-complexes and the "morphogenetic substances" of Acetabularia. Plant Science Letters. 3:297-302.

Avrameas, S. and T. Ternynck. 1969. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry. 6:53-66.

Barth, L. G. and L. J. Barth. 1959. Differentiation of the cells of the Rana pipiens gastrula in unconditioned medium. J. Embryol. Exp. Morphol. 7:210-222.

Berger, S., L. Sandakhchiev and H. G. Schweiger. 1974. Fine structural and biochemical markers of Dasycladaceae. Journal de Microscopie. 19:89-104.

Bonner, W. M. and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83-88.

Brachet, J. 1964. The role of nucleic acids and sulphydryl groups in morphogenesis (amphibian egg development, regeneration in Acetabularia). Adv. Morphogen. 3:247-300.

Brachet, J. 1967. Protein synthesis in the absence of the nucleus. Nature. 213:650-655.

Clemens, H. P. and W. H. Jones. 1954. Toxicity of brine water from oil wells. Transactions of the American Fisheries Society. 84:97-109.

Fraenkel-Conrat, H., B. Singer and A. Tsugita. 1961. Purification of viral RNA by means of bentonite. Virology. 14:54-58.

Godson, G. N. and R. L. Sinsheimer. 1967. Use of Brij lysis as a general method to prepare polyribosomes from Escherichia coli. Biochim. Biophys. Acta. 149:489-495.

Greenberg, J. R. 1977. Isolation of messenger ribonucleoproteins in cesium sulfate density gradients: Evidence that polyadenylated and non-polyadenylated messenger RNAs are associated with protein. J. Mol. Biol. 108:403-416.

Gurdon, J. B. 1968. Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. J. Embryol. Exp. Morphol. 20:401-414.

Gurdon, J. B., C. D. Lane, H. R. Woodland, and G. Marbaix. 1971. Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. Nature. 233:177-182.

Hämmerling, J. 1931. Entwicklung und Formbildungsvermögen von Acetabularia mediterranea. I. Die normale Entwicklung. Biol. Zentrabl. 51:633-647.

Hämmerling, J. 1932. Entwicklung und Formbildungsvermögen von Acetabularia mediterranea. II. Das Formbildungsvermögen kernhaltiger und kernloser Teilstücke. Biol. Zentrabl. 52:42-61.

Hämmerling, J. 1935. Über Genomwirkungen und Formbildungsfähigkeit bei Acetabularia. Roux' Arch. 132:424-462.

Hämmerling, J. 1963. Nucleo-cytoplasmic interactions in Acetabularia and other cells. Ann.Rev. Plant Physiol. 14:65-92.

Infante, A. A. and M. Nemer. 1968. Heterogenous ribonucleoprotein particles in the cytoplasm of sea urchin embryos. J. Mol. Biol. 32:543-565.

Jacob, F. and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3:318-356.

Keck, K. 1964. Culturing and experimental manipulation of Acetabularia. Pages 189-213 in D. M. Prescott, ed. Methods in Cell Physiology, Vol. 1. Academic Press, Inc., New York, New York.

Kloppstech, K. and H. G. Schweiger. 1975a. Nonribosomal ribonucleoprotein-particles in Acetabularia mediterranea. Cytobiologie. 12:1-12.

Kloppstech, K. and H. G. Schweiger. 1975b. Polyadenylated RNA from Acetabularia. Differentiation. 4:115-123.

Kloppstech, K. and H. G. Schweiger. 1976. In vitro translation of poly (A) RNA from Acetabularia. Cytobiologie. 13:394-400.

Kwapinski, J. B. G. 1972. Methodology of Immunochemical and Immunological Research. Page 319. Wiley-Inter-Science, Division of John Wiley and Sons, Inc., New York, New York.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680-685.

Laskey, R. A. and A. D. Mills. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56:335-341.

Maizel, J. V. 1971. Polyacrylamide gel electrophoresis of viral proteins. Methods Virol. 5:179-246.

Martin, D. W. and N. T. Owen. 1972. Repression and derepression of purine biosynthesis in mammalian hepatoma cells in culture. J. Biol.Chem. 247:5477-5485.

Müller, M. 1962. Uber jahres und lunarperiodische Erscheinungen bei einigen Braunalgen. Botannica Marina. 4:140-155.

Peacock, A. C. and C. W. Dingman. 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. Biochemistry. 7:668-674.

Reuter, W. and H. G. Schweiger. 1969. Kernkontrollierte Lactatdehydrogenase in Acetabularia. Protoplasma. 68:357-368.

Rowe, P. B. and J. B. Wyngaarden. 1966. The mechanism of dietary alterations in rat hepatic xanthine oxidase levels. J. Biol. Chem. 241:5571-5576.

Sandakhchiev, L., R. Niemann and H. G. Schweiger. 1973. Kinetics of changes of malic dehydrogenase isozyme pattern in different regions of Acetabularia hybrids. Protoplasma. 76:403-415.

Schweiger, H. G., R. W. P. Master and G. Werz. 1967. Nuclear control of a cytoplasmic enzyme in Acetabularia. Nature. 216: 554-557.

Schweiger, H. G., S. Berger, K. Kloppstech, K. Apel and M. Schweiger. 1974. Some fine structural and biochemical features of Acetabularia major (Chlorophyta, Dasycladaceae) grown in the laboratory. Phycologia. 13:11-20.

Schweiger, H. G. 1977. Transcription of the nuclear genome of Acetabularia. pgs. 65-83 in L. Bogorad and J. H. Weil, eds. Nucleic Acids and Protein Synthesis in Plants. Plenum Publishing Corp., New York, New York.

Shephard, D. C. 1969. Axenic culture of Acetabularia in synthetic media. Pages 49-69 in D. M. Prescott, ed. Methods in Cell Physiology, Vol. 4. Academic Press, Inc., New York, New York.

Spencer, T. and H. Harris. 1964. Regulation of enzyme synthesis in an enucleate cell. Biochem. J. 91:282-286.

Spirin, A. S. 1969. Informosomes. Eur. J. Biochem. 10:20-35.

Studier, F. W. 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. 79:237-248.

Szybalski, W. 1968. Use of cesium sulfate for equilibrium density gradient centrifugation. Pages 330-360 in L. Grossman and K. Moldave, eds. Methods in Enzymology, Vol. XII, part B. Academic Press, Inc., New York, New York.

Thomson, R. Y. 1969. Purines and pyrimidines and their derivatives. Pages 295-309 in I. Smith, ed. Chromatographic and Electrophoretic Techniques, Vol. 1. Interscience Publishers, Div. of John Wiley and Sons, Inc., New York, New York.

Werz, G. 1969. Morphogenetic processes in Acetabularia. Coll. Ges. Biol. Chem., Mosbach. 20:167-186.

Wilt, F. H., M. Anderson and E. Ekenberg. 1973. Centrifugation of nuclear ribonucleoprotein particles of sea urchin embryos in cesium sulfate. Biochem. 12:959-966.