Control of protein synthesis during the development of *Acetabularia*

By **GABRIEL CERON**\(^1\) AND **E. MARSHALL JOHNSON**\(^2\)

*From the Departamento de Morphologia, Universidad Nacional de Columbia and Curriculum of Human Morphology, California College of Medicine, University of California*

**SUMMARY**

Proteins from the soluble, chloroplastic and cell membrane fractions of axenically grown *Acetabularia* were analysed by zonaelectrophoresis. Incorporation of \(^{14}\text{C}\)leucine into different proteins was measured by autoradiographic analysis of the electrophoretic patterns. The protein patterns from the soluble fraction remain constant with respect to the number of detectable bands but change with respect to the relative synthetic rates at various developmental stages. The protein patterns from the membrane fraction change with respect to both the number of protein species and the relative synthetic rates. The analysis of the synthetic performance of enucleated cells revealed that most of the proteins from the soluble and the membrane fractions continue to be synthesized in the absence of the nucleus and that the changes that normally occur in the protein patterns of the membrane fraction at the time of cap formation also take place in enucleated cells. This is taken as an indication that the control of the synthesis of the proteins studied is of extranuclear nature.

It was also found that chloroplasts are capable of synthesizing all the components of the chloroplastic protein spectrum at least 4 weeks after enucleation. Some of the chloroplastic proteins can also be synthesized by purified chloroplasts in extracellular conditions.

The possibility of extranuclear control of protein synthesis being a rather general phenomenon during the development of *Acetabularia* is discussed.

**INTRODUCTION**

Development of the unicellular and uninucleate marine alga *Acetabularia* presents a series of problems in differentiation and morphogenesis which offer a favorable opportunity for the analysis of nucleocytoplasmic interactions during ontogeny. The analysis of such interactions at the molecular level is likely to give important clues as to the mechanisms that regulate gene expression in developing systems.

It is established that morphogenesis in *Acetabularia* is controlled by genetic information originated in the nucleus and accumulated in the cytoplasm where

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\(^1\) Author’s address: Departamento de Morphologia, Universidad Nacional de Colombia, Bogota, Columbia.

\(^2\) Author’s address: Curriculum of Human Morphology, California College of Medicine, University of California, Irvine, California 92664, U.S.A.
its expression takes place in a highly predictable and sequential manner (Hämmerling, 1931, 1934, 1953, 1963, 1966; Brachet, 1958, 1968; Werz, 1965). The information of the stalk always precedes the information of the apical cap; the latter, in turn, does not begin until stalk formation stops. The temporal sequence of these morphogenetic events does not depend upon sequential transcription of the nuclear genome since the information for both the stalk and cap formation is released simultaneously from the nucleus (Zetsche, 1964a, b, 1966a).

Thus, a sequential synthesis of stalk-specific or cap-specific proteins becomes the alternative explanation for the ordered realization of the morphogenic events. This possibility has been confirmed in various ways. From the studies of Clauss (1958), Brachet, Chantrenne & Vanderhaeghe (1955) and Hämmerling et al. (1958) it can be concluded that, in both normal and enucleated cells, not only protein synthesis is required for morphogenesis but synthesis of proteins must occur in an ordered fashion if normal stalk or cap formation is to take place. Although there is still no conclusive evidence for the existence of 'cap-specific' or 'stalk-specific' proteins, it has been demonstrated that inhibition of protein synthesis even for short periods leads to the production of stalk or cap abnormalities (Brachet, 1962, 1963; Brachet, Denis & de Vitry, 1964). Since the proteins required for cap formation are not likely to be chloroplastic gene products (Berger, 1967; Werz, 1965) it follows that enucleated cells are capable of controlling the orderly expression of nuclear genetic information.

The concept that control of differentiation in this system is carried out mainly by extranuclear mechanisms has gained considerable support from studies of the synthesis of certain enzymes related to cap morphogenesis. The activity of UDP-glucose-4-epimerase (Zetsche, 1966b), of alkaline phosphatase (Spencer & Harris, 1964) and of acid phosphatase isozymes (Triplett, Steens-Lievens & Baltus, 1965) normally increases at the time of cap morphogenesis. These changes also occur in enucleated fragments undergoing cap formation. This indicates that the mechanisms which control the expression of the genome during differentiation of Acetabularia operate at the level of translation (Brachet, 1967; Zetsche, 1966b; Spencer & Harris, 1964; Harris, 1968). This interpretation, however, applies only to those cases in which the increase in enzymic activity corresponds to an actual de novo synthesis of the enzyme (Zetsche, 1966b). Thus it is of considerable interest to learn if the same holds true for other proteins.

The purpose of the present study is to examine the ontogenetic patterns of a number of proteins from different subcellular fractions of Acetabularia, and to ascertain the extent to which the nucleus regulates their synthesis. This research was directed towards answering specific questions:

(1) Do protein synthetic patterns change with development?
(2) Are such patterns altered by enucleation?

These questions are posed to distinguish those proteins synthesized under
Control of protein synthesis in *Acetabularia* immediate nuclear control from proteins whose synthesis is directed by extranuclear mechanisms. Finally, we questioned nuclear regulation of protein synthesis by organelles such as the chloroplasts.

**MATERIALS AND METHODS**

*Culture of Acetabularia*

*Acetabularia crenulata* cells were cultured under axenic conditions following the methods of Lateur (1963) and of Gibor & Izawa (1963). The culture medium was prepared according to Keck (1964). Several cultures were maintained simultaneously, each one consisting of synchronously growing cells at a particular developmental stage. As a routine check for bacterial contamination, 2 mg/ml each of glucose and Tryptose (Difco) were periodically added to the medium. Turbidity indicated contamination in which case the cultures were discarded. To ensure bacteria-free experimental material, the cells were treated for 48 h immediately before the initiation of the labeling experiments with an antibiotic mixture: streptomycin, 200 mg, and penicillin, 200000 units, in 100 ml sterile sea water. Control experiments showed that this treatment permitted normal morphogenesis and regeneration of *Acetabularia* cells.

*Selection of developmental stages*

Seven developmental stages of *Acetabularia crenulata* were selected arbitrarily on the basis of the morphological appearance of the cells. These stages are defined as follows: 5 mm stage, cells averaging 5 mm in length and consisting of a rhizoid and a cylindrical stalk; 3 cm stage, cells averaging 3 cm in length; pre-cap stage, cells over 4 cm length consisting of a basal rhizoid and a cylindrical stalk with several whorls of filamentous processes at the apex of the stalk; cap primordium stage, cells over 4 cm in length bearing a club-shaped formation at the apex of the stalk; small cap stage, cells with a discoid cap not exceeding 3 mm in diameter; adult cap stage, cells with a full-grown apical cap usually 8 mm in diameter; mature cap stage, cells with a full-grown cap containing spherical spores in its radially oriented chambers.

*Enucleation procedures*

Enucleation was performed on cells at the 3 cm and cap primordium stages following standard surgical techniques for this organism (Keck, 1964). All operations were performed under sterile conditions. All enucleations for a given group (about 500 cells) were performed within 4 h. After a 12 h recovery period in the dark, the enucleated cells were placed in fresh medium and illuminated as usual.

*Labeling of Acetabularia cells*

Cells or cell fragments were incubated 36 h in Erdschreiber medium (Keck, 1964) containing 0-5 μCi/ml of [14C]-L-leucine with specific activity of 250 mCi/
The rate of incorporation of the label into *Acetabularia* proteins was determined by incubation of 100 pre-cap-stage cells for different periods of time and subsequent measurement of the specific activity of the TCA-precipitable material obtained from the soluble fraction (see Cell Fractionation section, below). Since the rate of incorporation was linear for at least 36 h, the incubation period for all subsequent labeling experiments was 36 h. During the labeling period the cells were kept under the standard illumination schedule.

**Cell fractionation**

Cells were fractionated at 4 °C following the method of Goffeau & Brachet (1965) with some modifications. After homogenization in mannitol-phosphate buffer, pH 6-8 (Goffeau & Brachet, 1965), the homogenate was filtered through a nylon mesh (pore size approximately 30 μm) mounted in a Swinnex-13 Millipore filter system. The filtrate was then centrifuged for 30 min at 20000 g and the resulting clear and colorless supernatant was taken as the soluble fraction for protein analysis and stored at −55 °C until needed to prepare the cell membrane fraction. Material retained in the filter was washed in 20 ml of mannitol-phosphate buffer and refiltered. The washed material was suspended in 20 ml of the same buffer and centrifuged for 10 min at 200 g. Convenient amounts of cell membrane and cell wall material were recovered and were consistently devoid of chloroplastic contamination upon microscopic examination. The membrane pellets were stored at −55 °C until needed for the extraction of the membrane-bound proteins.

**Extraction of membrane-bound proteins**

Washed membrane pellets were suspended in one volume of distilled water and two volumes of 9-0 M urea. The pH of the mixture was adjusted to 10 by addition of 0-1 M-KOH and incubated with occasional shaking for 12 h at 4 °C. After incubation the particular matter was separated by centrifugation at 10000 g for 20 min and the supernatant used for the analysis of the membrane proteins.

**Isolation of chloroplasts**

The procedure of Goffeau & Brachet (1965) for isolation of chloroplasts was followed in detail. The chloroplastic preparations were used for the study of chloroplastic proteins synthesized in normal and in enucleated cells as well as for the analysis of proteins synthesized by isolated chloroplasts. The isolation of the chloroplasts for the *in vitro* studies was carried out under sterile conditions. Glassware was autoclaved and homogenization buffer was sterilized by filtration through a Millipore filter (0-4 μm).
Labeling of isolated chloroplasts

Isolated chloroplasts were incubated according to Goffeau & Brachet (1965). [14C]leucine (specific activity of 250 mCi/mM) was used as the labeled precursor at a concentration of 0.1 μCi/ml of incubation mixture. The incubation time was at least 60 min, after which the chloroplasts were recovered by centrifugation at 1000 g and stored at −55 °C. The fractionation of the chloroplasts was done by freezing and thawing followed by ultrasonic vibration for 1 min at 4 °C. The resulting mixture was centrifuged at 20000 g for 30 min and the supernatant used for protein analysis.

Electrophoresis and autoradiography of Acetabularia proteins

Proteins from the soluble and the chloroplastic fractions were separated by disc electrophoresis in polyacrylamide gels (Ornstein, 1964; Davis, 1964). The concentration of acrylamide in the separation gel was always 9%. The sample gel was omitted and replaced by mixing the samples with 2.0 M-sucrose in a proportion of 3 parts of sample to 1 part of sucrose. Each gel was loaded with a total volume of 250 ml of sample-sucrose mixture containing 0.3–0.5 mg of protein. The protein concentration of all samples was determined by the Lowry method (Lowry, Rosenbrough, Farr & Randall, 1951). The amount of radioactivity in the form of TCA-precipitable material present in the samples was always 5 x 10⁴ c.p.m. The current for separation was 2 mA/gel at 4 °C. Electrophoresis was terminated when the Brom-phenol blue marker had migrated 7.5 cm. After electrophoresis the gels were fixed for 6 h in 15% TCA and stained with Coomassie brilliant blue (Chrambach, Reisfeld, Wyckoff & Zaccari, 1967). Electrophoretic analysis of proteins from the membrane fraction was carried out on a polyacrylamide system containing 8 M-urea in both the gels and the tank buffer. Electrophoresis was terminated when the dye marker had migrated 6.5 cm. After electrophoresis the urea-acrylamide gels were processed in the same manner as the standard gels.

Each assay was repeated with three different cultures of every stage studied. Each of these was subsequently divided into three parts for individual electrophoretic separation. The only differences, among the 9 gels of any experiment, were in the distances migrated. As can be seen in Fig. 5 and 6, these were remarkably minor even among the more rapidly migrating proteins.

After fixation, staining and photography of the protein patterns, the acrylamide gels were sliced longitudinally following the procedure developed by Fairbanks, Levinthal & Reeder (1965). The procedure for drying the acrylamide slices was simplified as follows: the center slices from each column were placed on shallow troughs made with glass plates precoated with a film of silicone and bordered with masking tape. Molten 1.5% de-ionized agar was poured over the slices, which had been previously aligned in the troughs, until evenly covered. After the agar had solidified, the plates were placed under a heat lamp for 1 h
and then allowed to dry under an ordinary desk lamp for 24 h. By this method the acrylamide slices were converted into perfectly even and smooth strips with a uniform thickness of 0.08 mm without distortions of the slices or band patterns. The dried slices were placed in contact with Kodak no-screen X-ray film (Fairbanks et al. 1965). The film was developed after 2 weeks of exposure and the resulting autoradiograms scanned with a Densicord apparatus (Photovolt, New York). Due to its simplicity and reproducibility the agar embedding method for drying the acrylamide slices was used routinely for the autoradiographic analysis of *Acetabularia* proteins.

**RESULTS**

*Protein synthesis during normal development*

Cells from various developmental stages were labeled with [14C]leucine and the proteins from the soluble and the membrane fractions studied. Autoradiographic analysis of proteins from the soluble fraction (Fig. 1) demonstrated that the synthetic patterns remain constant throughout development with respect to the number of electrophoretic components but change with respect to the relative synthetic rate of several proteins. These are exemplified by intensity of staining of bands at positions 0.6, 0.9, 2.3, 3.9, 4.4, and 5.5 from various developmental stages. The electrophoretic patterns of stainable proteins from the membrane fraction exhibit changes in both the number of the relative synthetic rates of various protein species (Fig. 2). For example, certain proteins appear *de novo* or their synthetic rate increases dramatically (bands marked *N* in Fig. 2) at the time of cap formation. Other proteins in turn seem to disappear at the time of cap morphogenesis (bands marked *X* in Fig. 2).

*Protein synthesis in enucleated cells*

The effect of enucleation upon the protein synthetic patterns during development was studied in two series of experiments. In the first series enucleation was performed on 3 cm stage cells and the proteins from the soluble and membrane fractions analysed 10 days and 6 weeks after enucleation. Ten days after enucleation the overall synthetic rate of the proteins from the soluble fraction is lower than in normal cells (Fig. 3). Six weeks after enucleation, however, the protein synthetic patterns for this fraction had essentially returned to normal levels. The electrophoretic patterns from the membrane fraction remain unaffected by enucleation (Fig. 4).

Six weeks after enucleation the cells exhibited the morphological characteristics of the cap primordium stage.

The second series of experiments included the study of the effect of enucleation upon the changes that normally occur in the synthetic pattern of the proteins from the membrane fraction at the time of cap morphogenesis. In this series, cells were enucleated at the cap primordium stage and the proteins from the
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Fig. 1. Electrophoretic patterns of proteins from the soluble fraction of selected developmental stages of *Acetabularia*. These zymograms are of gels stained for protein. The direction of migration was from the origin (−) at the top toward the bottom (+) of the figure. Distances migrated = scale in cm at the left. The stages examined were 5 mm, 3 cm, pre-cap (PC), small cap (SC), adult cap (AC), and mature cap (MC).

membrane fraction analysed when the enucleated cells had reached the adult cap stage. It was found that the changes that normally occur at this stage also take place in enucleated cells; that is, the proteins that normally disappear do so in the enucleated cells and the proteins that normally appear at this stage also appear in the enucleated cells (Fig. 5).
Analysis of chloroplastic proteins

The soluble proteins obtained from purified chloroplastic preparations were studied after their labeling in normal and enucleated cells as well as in isolated chloroplasts. In Fig. 6 it can be seen that both the staining and the autoradiographic patterns remain essentially unaffected even 4 weeks after the removal of the nucleus. The in vitro incubation and labeling of chloroplasts showed (Fig. 6) that some of the components of the chloroplastic protein spectrum can be synthesized in extracellular conditions.

DISCUSSION

The possibility that expression of genetic information may be controlled by extranuclear mechanisms and be of important mechanism of control during the differentiation of Acetabularia prompted the formulation of some of the questions considered in the present study. The question of whether the protein synthetic pattern in Acetabularia changes during its development can be answered affirmatively. The results presented above indicate that although most of the
proteins of the soluble fraction are present at all the developmental stages studied, their relative synthetic rates do change at different stages. Although the specific role of the different protein species present in this fraction cannot be inferred from the changes in their relative synthetic rates it is apparent that most of them are required throughout most of the vegetative phase of *Acetabularia* development. The slight decrease in the overall labeling of the proteins from the soluble fraction at the small cap and adult cap stages (Fig. 1) might be due to a

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**Fig. 3.** Autoradiographic patterns of proteins from the soluble fraction. *NC = normal cells at the 3 cm stage; 10-day enuc = cells examined 10 days after enucleation at the 3 cm stage; 6 wk enuc = cells examined 6 weeks after enucleation at the 3 cm stage. (See legend for Fig. 1.)**
decrease in the synthetic activity or in the absorptive capacity of the cell at these stages. This possibility, however, seems unlikely since the total amount of radioactivity in the form of TCA-precipitable material used on each gel is the same for all stages. It is possible that a considerable part of the radioactivity incorporated at these stages is diverted towards the construction of proteins that, although present in the soluble fraction, are not resolvable by this separation system due to their extremely high molecular weight or perhaps to a tendency to form aggregates that cannot penetrate into the gel system used in this study. It is also possible that part of the radioactivity present in the soluble fraction actually enters the gel but is distributed as radioactivity not resolvable as bands.

The changes observed in the synthetic patterns of the proteins from the membrane fraction at the time of cap formation are of considerable interest. These changes consist not only in the appearance of new components and the significant increase of others but also in the almost complete arrest of the detectable synthesis of certain protein species (Fig. 2). This is an example of a developmental change at the molecular level coincident with a developmental event detectable at the macroscopic level. The specific role of the proteins

\[ \text{Fig. 4. Autoradiographic patterns of proteins from the membrane fraction.} \]
\[ NC = \text{normal cells at the 3 cm stage; 10-day enuc = cell examined 10 days after enucleation at the 3 cm stage; 6 wk enuc = cells examined 6 weeks after enucleation at the 3 cm stage.} \]
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involved in these changes is not known. It is possible that they are enzymes involved in the metabolic changes that occur at the time of cap morphogenesis (Werz, 1965) or that they are structural proteins involved directly in the construction to the cap.

The synthetic performance of enucleated cells has been studied with regard to proteins from the soluble, the membrane, and the chloroplastic fractions. The

![Image of protein synthesis experiment](image)

Fig. 5. Protein synthesis in cells enucleated at the cap primordium stage. Stains and autoradiograms of proteins from the membrane fraction. Pre-cap, cap N and cap E = protein stain and autoradiograms of normal (nucleated) pre-cap and cap stages, and enucleated cells at the cap formation stage. N = component increasing or appearing during cap morphogenesis. X = components missing at the time of cap morphogenesis.

study of the proteins from the soluble fraction reveals that almost all the protein components resolvable by electrophoresis continue to be synthesized after the removal of the nucleus (Fig. 3). Ten days after enucleation only a few proteins of high molecular weight continue to be synthesized and most of the radioactivity is represented by elements of very low molecular weight. This phenomenon could be interpreted as being a residual effect of the surgical trauma
produced by enucleation rather than as a decrease in the number of RNA templates available for protein synthesis. This interpretation is supported by the fact that 6 weeks after enucleation the synthetic pattern of the proteins from this fraction appears almost completely restored. This is in agreement with earlier findings concerning the protein synthetic capabilities of enucleated cells (Clauss, 1958; Brachet, Chantrenne & Vanderhaeghe, 1955; Hämmerling et al. 1958) and suggests the existence, in *Acetabularia*, of messenger RNA molecules with a relatively long functional half-life. The effects of enucleation on the synthesis of proteins from the membrane fraction are similar to the ones described for the soluble fraction except that no apparent decrease in the labeling of the bands

![Fig. 6. Staining (S) and autoradiographic (A) patterns of chloroplastic proteins. Normal cells = cells at the 3 cm stage prior to enucleation. Enucleated cells = chloroplastic proteins 10 days and 4 weeks after enucleation performed on 3 cm cells. Extracellular synthesis = the arrows indicate proteins labeled under *in vitro* conditions (see text).](image-url)
Control of protein synthesis in Acetabularia occurs immediately following the removal of the nucleus. Thus it can be concluded that if nuclear in origin the messenger RNA for these proteins also has a long functional half-life.

When enucleation is performed at the cap primordium stage and the proteins from the membrane fraction isolated at later stages of cap morphogenesis (Fig. 5) it is found that the changes in the synthetic pattern under these conditions are the same as those occurring at the time of cap formation in normal cells. This is an example of a sequential synthetic event taking place in the absence of the nucleus and can be regarded as supporting evidence for the idea proposed by Spencer and Harris that the control of protein synthesis during cap morphogenesis is exerted at the level of translation rather than at the level of transcription.

Because of similarities in synthetic patterns, it might appear that the protein components appearing in the membrane fraction at the time of cap formation in both normal and enucleated cells are similar to the enzymes studied by Zetsche (1966b) and by Spencer & Harris (1964). This is probably not the case since the enzymes studied by these authors belong to the soluble rather than to the membrane fraction for two reasons: first, the fractionation methods employed in the isolation of alkaline phosphatase (Spencer & Harris, 1964) and of uridine-diphosphate glucose 4-epimerase (Zetsche, 1966b) dispose of the membrane fraction, leaving only the high-speed centrifugation supernatant for enzyme analysis; secondly, the method of extraction of proteins from the membrane fraction with 8.0 M-urea as employed in this investigation has not been reported before, making it more likely that such proteins belong to a class not previously described and whose specific role is still unknown.

The general conclusions that can be drawn from the study of protein synthesis in enucleated cells are: that the synthesis of most of the protein components of the soluble and the membrane fractions does not depend upon immediate nuclear control, that the sequential changes in the synthesis of certain proteins from the membrane fraction can indeed take place in enucleated cells and, finally, that since it is unlikely that all these proteins are coded by chloroplastic genes, the genetic messages for at least some of them must come from the nucleus, in the form of long-lived messenger RNA.

These findings demonstrate that the extranuclear nature of the control of protein synthesis in Acetabularia is a rather general phenomenon. If, as proposed by Spencer & Harris (1964) and others, translational control of protein synthesis plays a fundamental role in the differentiation of eukaryotes, then Acetabularia becomes one of the organisms of choice for the study of this important developmental problem.

The problem of translational control in Acetabularia has been discussed mainly by Zetsche (1966a, b), Spencer & Harris (1964) and Harris (1968). According to these authors the stability of the messenger RNA that codes for the proteins involved in cap formation is demonstrated by the fact that enu-
Cleated apical stalk fragments can be kept in the dark for several weeks or in the presence of protein inhibitors for several days and yet, when such fragments are placed under optimal conditions for growth, the formation of the cap still takes place. Very little is known of the physical characteristics of the messenger since the attempts to isolate any kind of messenger RNA from *Acetabularia* have been unsuccessful. The long half-life of the messenger RNA for cap proteins cannot be due to the absence of ribonucleases from the cytoplasm (Schweiger, 1966), since one of the problems in the isolation of undegraded RNA from *Acetabularia* is the presence of extremely active ribonucleases (Brachet, 1968; Farber, 1969a, b).

The problem of the molecular genetic autonomy of chloroplasts has been studied with regard to protein synthesis. The results reported here indicate that the protein synthetic performance of chloroplasts is not modified to any detectable extent by the removal of the nucleus. The synthetic patterns of chloroplastic proteins obtained 4 weeks after enucleation are indistinguishable from those of normal controls. Enucleation has been found to alter the morphology and the reproductive behavior of chloroplasts in *Acetabularia* (Sheppard, 1965b), but produces no appreciable effect upon the overall incorporation of protein precursors by the chloroplasts (Sheppard, 1965a). The results of this investigation confirm the latter observation and suggest that either the proteins detected by the present method are truly chloroplastic-gene products independent from nuclear control, or that, if the genetic information for some of the chloroplastic proteins is originated in the nucleus, it has a functional half-life of at least 4 weeks. In order to distinguish between those two alternatives, the attempt has been made to determine which of the proteins isolated from the chloroplasts are synthesized by these organelles in isolation as a preliminary step towards the detailed study of the origin and half-life of genetic messages. The protein species synthesized by isolated chloroplasts under the conditions described here represent only a small part of the normal protein spectrum. This does not indicate that these organelles are limited in their synthetic capacity to those protein species detected in the present in vitro studies. Further research is needed in order to establish clearly the extent of the interactions between the nucleus and these organelles.

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