

STUDIES ON *ACETABULARIA* CHLOROPLAST DNA

by

BERNICE L. MUIR

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Department of Botany

The University of British Columbia
Vancouver 8, Canada

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ABSTRACT

The physical properties and renaturation kinetics of DNA extracted from isolated chloroplasts of *Acetabularia mediterranea* has been studied. It has a buoyant density of 1.702 g/cm^3 , which corresponds to a base composition of 42.8% G+C. When melted in SSC, *Acetabularia* chloroplast DNA has a T_m of 86.7° , corresponding to a base composition of 43% G+C. The close agreement of the base compositions calculated from the buoyant density and the melting temperature indicates the absence of unusual bases in *Acetabularia mediterranea* chloroplast DNA.

In $0.1 \times \text{SSC}$, *Acetabularia* chloroplast DNA melts with a T_m of 70.7° , and the melting transition is very broad. The breadth of the melting transition suggests that this DNA has a high degree of intramolecular heterogeneity. A differential plot of the thermal transition of *A. mediterranea* chloroplast DNA supports this conclusion.

The buoyant densities of DNA from bacterial contaminants found in *Acetabularia* cultures differed from the buoyant density of the chloroplast DNA. In any case, the amount of bacterial contamination was too low to account for any of the results obtained.

Renaturation experiments indicate a kinetic complexity of 1.1×10^9 daltons from *Acetabularia mediterranea* chloroplast DNA. As a result of uncertainties in the values of alkaline sedimentation coefficients, this calculated kinetic complexity may be too low.

The possible genetic information contained in the chloroplast DNA of *Acetabularia mediterranea* is discussed.

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INTRODUCTION

The purpose of this research was to determine the complexity of *Acetabularia mediterranea* chloroplast DNA from its renaturation kinetics. *Acetabularia mediterranea* has been used extensively to study nucleocytoplasmic interactions and, thus, it is of interest to determine something about the genetic potential of the chloroplast DNA. Also, with *Acetabularia* it is possible to obtain chloroplast DNA which is completely free from contamination by nuclear DNA. For these reasons, *Acetabularia mediterranea* was chosen for this research.

Renaturation kinetics are useful for studying the complexity and, therefore, the genetic potential of DNA from a particular source. DNA may be denatured by heat, alkali or various chemicals, resulting in complete separation of the complementary strands. Under appropriate conditions of temperature, ionic strength and pH, the separated complementary strands come back together in register, i.e. renature. The conditions for renaturation and the kinetics of the reaction have been studied extensively by various researchers (12,13,14,40,45,65,68,74) and may be summarized as follows:

- 1) The renaturation reaction is a second order process
- 2) The second-order rate constant for renaturation is inversely proportional to the complexity of the DNA, where complexity is defined as the total number of DNA base pairs in non-repeating sequences.
- 3) The optimum temperature for renaturation is 20° to 30°C below the melting temperature (T_m) of the DNA.

- 4) The DNA renaturation rate is dependent on ionic strength of the solvent for an electrolyte such as NaCl.
- 5) Decreasing the molecular weight of a given DNA results in a decrease in the rate of renaturation of the DNA.
- 6) The rate of renaturation increases slightly with increasing GC content.
- 7) The rate of renaturation of DNA depends on solvent viscosity.
- 8) Within the pH range 5 to 9 in 0.4 M Na⁺ the rate of renaturation is essentially independent of pH.
- 9) Correct base sequence matching can occur to a greater extent when the DNA is sheared to low molecular weight.
- 10) The thermal stability of the DNA is decreased about one degree for 1.5 per cent mispaired bases in the renatured DNA.

Wetmur and Davidson (74) have derived the following equation for determining the kinetic complexity of DNA.

$$N_D = 331N = \frac{5.5 \times 10^8 (S_{20,w}^{pH13})^{1.25}}{k_2}$$

where N_D is the complexity of the DNA in daltons, k_2 is the second order rate constant for renaturation of the DNA; and $S_{20,w}^{pH13}$ is the alkaline sedimentation coefficient of the DNA. This equation is valid when the renaturation rate constant is determined at $[Na^+] = 1.0 \text{ mole l}^{-1}$ in aqueous solution at a temperature near $(T_m - 25)^\circ\text{C}$.

Acetabularia has been an important research organism for two reasons. Since the 1930's, this marine alga has been used extensively to study

nucleo-cytoplasmic interactions. More recently, *Acetabularia* became the first plant in which it was possible to demonstrate the existence of DNA in the chloroplasts.

With *Acetabularia mediterranea*, a single cell may achieve a length of 3 to 5 cm in a period of 3 months. During this vegetative part of the life cycle a single, large nucleus is located in the rhizoid at the base of the cell. Growth of the cell culminates in the formation of a reproductive cap, which takes about one month. The diameter of the stalk at this time may be 0.3 to 0.5 mm, while the full grown cap may attain a diameter of up to one cm. When the cap is fully grown, the large primary nucleus divides to form several thousand small, secondary nuclei. By means of cytoplasmic streaming, the secondary nuclei are transported from the rhizoid to the cap, where cysts are formed (33,48). Thus, *Acetabularia* can readily be enucleated by excising the rhizoid before the cell forms a cap.

If immature *Acetabularia* cells are cut to produce a nucleate, rhizoid portion and an anucleate stalk portion, not only can the nucleate rhizoid regenerate a complete new plant, but the anucleate stalk can survive for several weeks and continue to grow. In some cases the anucleate stalk will even differentiate a cap. This and other findings led Hämmerling to postulate the existence of "morphogenetic substances" which are produced by the nucleus but may be present in the cytoplasm for long periods of time (33). Excision and interspecific grafting experiments have shown that these "morphogenetic substances" induce cap formation and are necessary for differentiation of a cap with species-specific morphology (33).

Within a single *Acetabularia* cell are several million chloroplasts.

The number of chloroplasts increases exponentially during normal growth of the cell, with the chloroplast doubling time being a little longer than one week (55). The chloroplasts increase in number in anucleate fragments, also, but in this case the doubling time is about two weeks (55).

The presence of DNA in the chloroplasts of *Acetabularia* was shown in 1963 (4,27). This was the first instance in which DNA could be definitely attributed to the chloroplasts without the possibility of contamination by nuclear DNA. The presence of DNA in the chloroplasts leads to speculation about its possible role in directing the activities of the cell. Knowledge of the amount of DNA in the chloroplasts and the presence or absence of repeated nucleotide sequences can give an indication of the genetic potential of the chloroplast DNA.

The situation is complicated by observations that the amount of DNA per chloroplast is variable (74). Using several techniques, Woodcock and Bogorad (75) detected large, but variable, amounts of DNA in only 35% of the chloroplasts. Similarly, Green and Burton (30) found DNA associated with only 20-40% of chloroplasts osmotically shocked by the Kleinschmidt technique and observed with the electron microscope. Green and Burton (30) attribute this to an intrachloroplastal nuclease. In spite of these observations, however, estimates of the average amount of DNA per chloroplast can be made.

Using fluorometric analyses, Gibor and Izawa (27) estimated the amount of DNA to be 1×10^{-16} g per chloroplast. This is comparable to the amount of DNA in a virus. Observation of *Acetabularia* chloroplast DNA with the electron microscope has shown, however, that at least some chloroplasts

contain much larger amounts of DNA (30,31,75). This suggests that the estimate of Gibor and Izawa is too low. More recently, using a scaled down diphenylamine assay for DNA, Green determined that the average amount of DNA per chloroplast is about 2.3×10^{-15} g (unpublished). This is comparable to the amount of DNA in the genomes of a number of bacterial species, including *Achromobacter anitratus*, *Streptococcus faecalis*, *Diplococcus pneumoniae* and *Staphylococcus aureus* (3). Gibor and Izawa estimated the amount of DNA per chloroplast from the protein to DNA ratio. Their calculation of the amount of protein per chloroplast was based on the assumption that proteins comprise 20% of the organelles. Green did actual counts of the number of chloroplasts. Perhaps this accounts for the discrepancy between the two results. Determination of the kinetic complexity of the chloroplast DNA should help to resolve this.

A prerequisite for the DNA renaturation reaction is that the DNA be double stranded. Evidence for the double strandedness of *Acetabularia mediterranea* chloroplast DNA comes from electron microscopy (31,73) and from buoyant density studies of chloroplast DNA which has been heat denatured (32). Thus, it is possible to study the renaturation kinetics of the chloroplast DNA. The formula of Wetmur and Davidson (74) can then be used to determine the kinetic complexity of *Acetabularia* chloroplast DNA.

To study the renaturation kinetics, it is necessary to obtain relatively pure chloroplast DNA. With *Acetabularia*, contamination by nuclear DNA can be ruled out by excising the rhizoids, which contain the nuclei, and isolating chloroplasts only from the stalks of immature cells. It is more difficult to rule out the possibility of contamination by mitochondrial DNA, but the amount of DNA in a mitochondrion appears to be less than in a

chloroplast (75). Therefore, many mitochondria have to be present in a chloroplast preparation in order to contribute a significant amount of DNA. Such an amount will show up as a second UV absorbing band at $\rho = 1.714 \text{ g/cm}^3$ in a cesium chloride density gradient (32). Heilporn and Limbosch (34) find that the mitochondrial DNA renatures faster than the chloroplast DNA. Therefore, mitochondrial DNA, if present, may be detected as a faster renaturing component.

Axenic cultures of *Acetabularia* are difficult to maintain, so that contamination with bacterial DNA can be a problem. This can be minimized by treating the *Acetabularia* cells with antibiotics before use, and by selective techniques during the chloroplast isolation. The chloroplast preparations can be monitored to determine the amount of bacterial contamination. If necessary, the chloroplast DNA can be separated from bacterial DNA by centrifuging in a CsCl density gradient, provided that the bacterial DNA has a different buoyant density than the chloroplast DNA. Unfortunately, there has been disagreement about the buoyant density of the chloroplast DNA as reported in the literature (9, 26, 32, 34). In the process of this research, this problem was resolved. A buoyant density of 1.702 g/cm^3 was found for *Acetabularia* chloroplast DNA. This agrees quite closely with the value given by Green et al. (32) and Heilporn and Limbosch (34).

Whatever the amount of DNA in the chloroplasts, it is not very great and, thus, a major difficulty of this research is to obtain large enough quantities of DNA to study the renaturation kinetics. The relatively slow growth of *Acetabularia*, and the fact that each cell has to be handled individually to enucleate it, are further limitations to preparing large amounts of *Acetabularia* chloroplast DNA.

MATERIALS AND METHODS

The following abbreviations are used: BSA, bovine serum albumin; EDTA, disodium ethylenediamine tetraacetate; SDS, sodium dodecyl sulphate; SSC, standard saline citrate (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0); TES, N-tris (hydroxymethyl)methyl-2-aminoethane sulfonic acid; Tris, tris (hydroxymethyl)aminomethane.

Materials

Reagent grade chemicals were used throughout. Ficoll was purchased from Pharmacia (Uppsala, Sweden). Pentex brand BSA was obtained from Miles Laboratories. TES was purchased from the Nutritional Biochemical Company but contained large amounts of impurities. Therefore, later supplies of TES were obtained from Calbiochem. Mallinckrodt phenol or Fisher liquified phenol were used. Pancreatic ribonuclease and lysozyme were purchased from Worthington Biochemical Corp., while pronase and α -amylase were obtained from Calbiochem. Ribonuclease T₁ was purchased from the Nutritional Biochemical Company. Aquacide II is a product of Calbiochem. Optical grade CsCl was purchased from Schwarz Bioresearch Inc.

Treatment of cysts prior to germination:

Acetabularia cysts are resistant to killing by some agents which will kill growing cells. Thus, in an attempt to obtain axenic cultures, cysts were treated in several ways. All solutions and equipment were sterilized before use and sterile technique was used throughout the procedures.

Ripe caps which had been stored in seawater in the dark at 10° for several months were washed with seawater and cut with scissors to release

the cysts. (In some cases, Shephard's artificial seawater medium (57) was used in place of seawater). To separate cell debris from cysts, the cut caps were filtered on 123 μ mesh bolting silk, washed with sea water and the cysts drawn through by gentle suction. The cysts settled to the bottom of the flask after one to two minutes and the seawater was decanted. Fresh seawater was added and the contents of the flask shaken for one or two minutes. After the cysts settled, the seawater was decanted. This washing procedure was repeated five or six times. The cysts were then treated in one of the following ways:

- 1) The cysts were placed in a small volume of 1% SDS in distilled water and the mixture agitated for one hour with a small magnetic stirring bar. The cysts were then allowed to settle and the SDS solution was poured off. Following this, the cysts were washed seven times with seawater, each time letting the cysts settle and removing the seawater by decantation or by carefully withdrawing it with a Pasteur pipette. Then the cysts were placed in 2 ml of enriched seawater medium or a mixture of enriched seawater and Shephard's medium and left in the light at 20° to germinate. (Enriched seawater medium is Shephard's medium in which seawater with NaNO_3 0.04 g/l and K_2HPO_4 0.001 g/l added, replaces the macronutrient salt solution. Micronutrient salts, NaHCO_3 and vitamins are added as for Shephard's medium).
- 2) The cysts were treated with SDS as above, then placed in nutrient Shephard's medium (Shephard's medium with glucose 0.6 g/l and tryptone 0.6 g/l added) with penicillin 1 mg/ml, neomycin 100 $\mu\text{g}/\text{ml}$ and streptomycin 100 $\mu\text{g}/\text{ml}$ in the dark at 20°. After two days, the cysts were

washed twice with seawater and placed in enriched seawater in the light to germinate.

- 3) The cysts (some with, and some without, prior treatment with SDS as above), were treated with lysozyme 1 mg/ml in 0.001 M Tris, 0.001 M EDTA, pH 7.0, at room temperature for 20 minutes. They were washed twice with seawater, then treated with 1% SDS in distilled water for 15 minutes. After being washed 5 to 10 times with seawater, the cysts were placed in 2 ml enriched seawater in the light to germinate. In some cases the cysts were treated, as above, with antibiotics in the dark for two days before being placed in the light to germinate.
- 4) After treatment with SDS as above, the cysts were treated with 10% Argyrol in seawater (27) for 20 minutes. Following this, the cysts were collected on 25 μ mesh bolting silk (held in a Millipore filter apparatus), washed with 50 ml seawater, transferred to a test tube and washed three more times with seawater. Then they were put into 2 ml nutrient Shephard's medium with penicillin 1 mg/ml and streptomycin 100 μ g/ml in the dark for two days. Following this, the cysts were washed three times with seawater and placed in 2 ml Shephard's medium in the light to germinate.
- 5) The cysts were treated with 10% Argyrol in Shephard's medium for 20 minutes, collected on 25 μ mesh bolting silk and washed with 150 ml Shephard's medium. They were then transferred to a test tube and washed five times with Shephard's medium. Following this, the cysts were treated with 1% SDS in distilled water for 15 minutes, then washed seven times with Shephard's medium. Finally, the cysts were treated with antibiotics as above before being left in the light to germinate.

Cultivation and treatment of algae

Acetabularia mediterranea were cultivated in 500 ml Bellco flasks containing 200 ml sterile Shephard's medium (57), with approximately 200 cells per flask. They were grown at 20°C and illuminated with 250-300 ft candles of fluorescent light for 12 hr/day. Some of the cells were grown at a lower light intensity in an attempt to inhibit cap formation (48).

For 2 to 5 days before use, cells were treated with one or more of the following antibiotics, at the concentrations indicated: penicillin 1 mg/ml, kanamycin 100 µg/ml, neomycin 100 µg/ml, streptomycin 100 µg/ml or chloramphenicol 10-50 µg/ml. When streptomycin or chloramphenicol were used, the cells were placed in the dark to prevent damage to the chloroplasts. Cells were also placed in the dark to reduce the amount of starch in the chloroplasts and thus prevent chloroplast breakage during the isolation procedure. The usual regime was to wash the cells two or three times and place them in fresh medium with neomycin for two days. Then the cells were washed twice and placed in fresh medium with penicillin and kanamycin or streptomycin for two days in the dark. The cells were then used to prepare chloroplasts.

Isolation of Chloroplasts:

Sterile technique was used throughout. Equipment and glassware were sterilized in an autoclave or oven. Solutions were sterilized by passage through a 0.22 µ Millipore filter.

Cells two to five cm in length were used, prior to cap formation. In a few cases, cells with cap initials less than 1.5 mm in diameter were used.

The cells were washed three times and each cell enucleated by cutting off the rhizoid along with 2 to 3 mm of the stalk, using iridectomy scissors. In a few instances, the nucleate fragments were collected and placed in fresh medium to regenerate new stalks. After a recovery period of one or two hours in dim light, the stalks were collected and used. In preliminary preparations, Shephard's method was used to isolate the chloroplasts (59). This was modified slightly for later preparations. All operations were carried out at 0-4°. Centrifuging was done in the swinging bucket rotor of a Sorvall R2B centrifuge.

Approximately 2 g wet weight of enucleated cells were scissor minced in 3 ml of a medium consisting of 0.6 M mannitol, 0.1% BSA, 0.001 M EDTA, 0.1 M TES and 0.001 M dithiothreitol, pH 7.8 (H medium). The slurry was filtered through 25 μ mesh bolting silk under gentle suction and washed through with approximately 3 ml of a medium similar to H but containing 0.01 M TES (W medium). Cell wall debris was retained by the bolting silk, while the chloroplasts and cytoplasmic content passed through. The chloroplast suspension was layered onto 4 ml of W medium with 2% Ficoll added (WF medium) and centrifuged for 15 min at 650 x g. The pale green supernatant was discarded. The chloroplast pellet was suspended in 4 ml W medium, layered over 4 ml WF medium and centrifuged at approximately 90 x g for 5 minutes. The very small green and white pellet was discarded and the green supernatant was passed through a 5 μ Nucleopore filter to break up chloroplast aggregates and shear off tags of cytoplasm adhering to the chloroplasts. This step also removed a significant number of bacteria. The filtrate containing the chloroplasts was then centrifuged at 650 x g for 15 minutes and the pale green supernatant discarded. The chloroplast pellet was

suspended in 4 ml W medium, layered over 4 ml WF medium and centrifuged at 650 x g for 15 minutes. (This step was sometimes omitted). The pale green supernatant was discarded. The chloroplast pellet was suspended in 5 ml of a medium containing 0.6 M mannitol, 0.1% BSA, 0.025 M TES, 0.01 M KCl, 5 mM MgCl₂ and 0.5 mM KH₂PO₄, pH 7.8 (A medium) and centrifuged at 650 x g for 15 minutes. The very pale green or clear supernatant was discarded and the chloroplast pellet resuspended in A medium. Depending on the amount of starting material, from one to four pellets were combined and suspended in a final volume of 6.6 to 16.6 ml of A medium. After thorough mixing of the chloroplasts, 0.6 ml was taken to assay for bacterial contamination and the remaining chloroplast suspension was centrifuged at 650 x g for 15 minutes. The supernatant was discarded and the pellet containing 90-95% intact chloroplasts, was used for the preparation of DNA.

In a few preliminary preparations, instead of suspending the chloroplasts in A medium in the final steps, the chloroplast pellet was suspended in 4 ml W medium and layered over 4 ml 0.8 M sucrose in 0.005 M EDTA, 0.01 M TES, 0.001 M dithiothreitol, pH 7.8. The suspension was centrifuged at 650 x g for 15 minutes. Sometimes this was not adequate to pellet the chloroplasts, in which case centrifugation was repeated at 1000 x g. The chloroplast pellet was then suspended in 5 ml 0.4 M sucrose buffered as above, and 0.7 ml taken for contamination check. The remaining suspension was centrifuged at 650 x g for 15 minutes and the chloroplast pellet used for preparation of DNA.

Preparation of chloroplast DNA

In preliminary experiments, DNA was prepared by a slight modification of the phenol-pH9-RNases method of Miura (46). The chloroplast pellet from

2-4 g wet weight of *Acetabularia* cells was suspended in 2-3 ml tris-SDS-buffer (0.1 M Tris, 1% SDS, 0.1 M NaCl, pH 9.0) and left on ice for 10-15 min for lysis to occur. An equal volume of phenol saturated with tris-SDS buffer was added and the mixture shaken in the cold for 20 min. Layers were separated by centrifugation and the clear, upper, aqueous layer carefully pipetted off. Since there was not enough DNA to be precipitated with ethanol, the aqueous layer was dialyzed against 2 l 0.1 x SSC (3 changes) in the cold for 24 to 48 hours. DNA from several preparations was pooled and stored in the refrigerator (4°) with a drop of chloroform. The volume of the pooled samples was reduced with Aquacide II and the DNA solution incubated at 37° with ribonuclease T₁ 25-100 units/ml and pancreatic ribonuclease 50 µg/ml for 30-45 min. Pronase (free of deoxyribonuclease by the method of Stern (62)) 100 µg/ml was added and the solution incubated at 50° for one to two hours. An additional 50 µg pronase/ml was added and the incubation continued for 2 to 3 hours. The solution was then dialysed against 2 l SSC (2-3 changes) in the cold and concentrated with Aquacide II. The DNA solution was adjusted to a density of 1.700 g/cm³ by the addition of solid CsCl and centrifuged at 42000 rpm in the 50 Ti rotor of a Beckman model L2B ultracentrifuge for 61 hr at 20°. The bottom of the centrifuge tube was punctured with a 27 gauge needle and 4 drop fractions were collected. Each fraction was diluted with 0.5 ml 0.1 x SSC and the OD_{260nm} monitored. Peak fractions containing DNA of $\rho = 1.702 \text{ g/cm}^3$ were pooled and dialysed into SSC in the cold.

In later experiments, a slight modification of Marmur's method (44) was used to prepare DNA from the chloroplasts. The chloroplast pellet was suspended in 1.25 ml 0.15 M NaCl₂, 0.1 M EDTA, pH 8 and 0.1 ml 25% SDS was

added. The mixture was heated to 60° for 10 minutes and then chilled on ice. Following the addition of 0.33 ml 5 M NaClO_4 , one volume of chloroform:isoamyl alcohol (24:1 V/V) was added and the mixture shaken in the cold for 20 minutes. The layers were separated by centrifugation. The clear, upper, aqueous layer containing the DNA was carefully withdrawn and dialysed against 2 l SSC (2 changes) in the cold in the dark for 24 hours. After dialysis the DNA solution was stored in the freezer (-20°). DNA from several preparations was thawed and pooled, then treated at 37° with pancreatic ribonuclease 100 $\mu\text{g/ml}$ and ribonuclease T_1 25-50 units/ml for 30 minutes, and α -amylase 5-10 $\mu\text{g/ml}$ for 15 minutes. Pronase 100 $\mu\text{g/ml}$ was added and the incubation carried out at 50° . After one to two hours, pronase 50 $\mu\text{g/ml}$ was added and the incubation continued for 2 to 3-1/2 hours. The solution was chilled on ice and then dialysed overnight against 2 l SSC in the cold in the dark, concentrated with Aquacide II, and dialysed a further 6 to 18 hr against 2 l SSC in the cold in the dark. Treatment with the ribonucleases, α -amylase and pronase, as above, was repeated and the DNA solution was again dialysed against SSC. After further reduction of the volume with Aquacide II, the DNA solution was clarified by centrifuging at $18000 \times g$ for 10 min. A small white pellet was discarded and the clear supernatant was dialysed into SSC or 0.1 x SSC.

Monitoring for bacterial contamination

To check the effectiveness of various treatments of cysts, aliquots of the final wash, along with a few cysts, were plated on nutrient Shephard's agar (nutrient Shephard's medium solidified with 1.5% agar) with a soft agar overlay and incubated at 20° . After one week colonies were counted and contaminants, when present, were characterized.

Occasionally, individual *Acetabularia* cells were observed with a phase microscope to check for the presence of bacteria and other protists. At several times during the growth phase, *Acetabularia* cells were plated on nutrient Shephard's agar along with a small amount of the medium in which they were growing. Plates were incubated at 20° and the nature and extent of bacterial growth was noted.

To check for contamination in chloroplast preparations, aliquots of the final chloroplast suspension were added to nutrient Shephard's medium to make final dilutions of 10^{-1} , 10^{-2} and 10^{-3} . Five tubes of each dilution were incubated at 20° and the number of samples of each dilution which became turbid after one week were counted. Most probable numbers of bacteria were estimated from the table given by Collins (17). In addition to this, aliquots of each dilution were plated on nutrient Shephard's agar. Colonies were counted and characterized after incubation at 20° for one week. Chloroplast numbers were determined by counting two aliquots of a 10^{-1} dilution in a hemacytometer. The ratio of bacteria to chloroplasts and thus the amount of DNA being contributed by bacteria could then be estimated.

Isolation of DNA from bacterial contaminants

Bacteria were grown in nutrient Shephard's medium overnight with shaking at 25°. Cells were harvested by centrifugation and DNA extracted according to the phenol-pH9-RNases method of Miura (46). Freezing and thawing was not necessary to lyse the cells, but in some cases pronase (free of deoxyribonuclease (62) 100 µg/ml was used to disrupt filaments.

Cesium chloride density gradients

About 0.8 ml of the solution containing 1-5 μ g DNA/ml was adjusted to a density of 1.70 g/cm³ by the addition of solid CsCl. *Micrococcus lysodeikticus* DNA (ρ = 1.731 g/cm³) was added as a density reference. The solution was centrifuged at 44,000 rpm in a 12 mm Kel-F cell in the AN-D rotor of a Spinco Model E at 20°. Photographs were taken after 20 hr and the negatives scanned with a Joyce-Loebl microdensitometer. Buoyant densities were calculated according to Mandel et al. (43), without correction for pressure affects.

Electron microscopy

Chloroplasts were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer, pH 6.8, and post-fixed in 1% OsO₄ in cacodylate buffer. After dehydrating with an ethanol series, the chloroplasts were infiltrated with, and embedded in, Spurr's medium (60) in gelatin capsules; cured in a vacuum oven at 70° for 10 hours and thin sectioned. Silver sections were collected on 200 mesh copper grids and stained with uranyl acetate followed by lead citrate. Sections were examined with an Hitachi HU 11-A or HS-7S electron microscope.

Preparation of *E. coli* DNA

E. coli K 12 was obtained from Dr. R. Warren of the Microbiology Department, U.B.C. Cells were grown in nutrient broth (Difco) with 5% yeast extract (Difco), pH 7.5, at 30° or 37° with shaking and harvested by centrifugation. DNA was extracted by the method of Miura (46), with several additional purification steps. Following treatment with pancreatic ribonuclease 50 μ g/ml and ribonuclease T₁ 25 units/ml at 37° for 30 minutes, the DNA

in SSC was incubated with pronase (deoxyribonuclease free) 100 $\mu\text{g/ml}$ at 50° for one to two hr. Pronase 50 $\mu\text{g/ml}$ was added and the treatment continued for 2 to 3 hours. The solution was chilled and 2 volumes of cold ethanol added. DNA fibers were collected on a glass rod, washed in ethanol and dissolved in 0.1 M sodium acetate buffer, pH 6.0. The solution was adjusted to 0.13 M sodium acetate by addition of 3 M sodium acetate and one volume of ethoxyethanol was added to precipitate the DNA. This step separates the DNA from ribonucleotides (35). Kirby's two-phase method, as described by Bellamy and Ralph (7), was used to remove polysaccharides. The DNA was dissolved in 0.025 M Tris-HCl buffer, pH 8.1, containing 0.025 M NaCl. One volume cold 2.5 M phosphate buffer, pH 8, and one volume cold 2-methoxyethanol were added and the mixture shaken vigorously for 3 minutes at 4° . After centrifuging at $12,000 \times g$ for 5 minutes, the clear upper layer was carefully withdrawn and two volumes of cold ethanol added to it. The DNA precipitate was dissolved in 0.1 x SSC and the solution stored in the refrigerator (4°) with a drop of chloroform.

Preparation of bacteriophage T4 DNA

Purified phage T4 particles were a gift of Dr. R. Miller of the Microbiology department, U.B.C. The phage particles in 0.5 ml of 0.01 M Tris, 0.15 M NaCl, pH 7.5, were shaken with an equal volume of water saturated phenol for 15 min at 4° . The mixture was centrifuged at $2000 \times g$ for 10 min to separate the layers. The clear upper aqueous layer was carefully pipetted off and dialysed against 2 l of SSC (2 changes) in the cold for 24 hrs. The DNA solution was then stored in the refrigerator (4°) with a drop of chloroform.

Thermal denaturation profiles and determination of T_m

Solutions of DNA in SSC or 0.1 x SSC were degassed by evacuation in a vacuum dessicator. Spectrosil semi-micro cuvettes fitted with teflon stoppers were used and the OD_{260nm} automatically recorded with a Gilford 2400 spectrophotometer. The temperature in the chamber was increased rapidly to about 50° and then at a rate of 1° per 4 min until the onset of melting. After that point, the temperature was increased 1° per 10 min until maximum hyperchromicity was reached. Absorbance measurements were corrected for thermal expansion of the solvent and the T_m determined from the plot of relative absorbance (A_T/A_{250}) versus temperature (42). A sample of *E. coli* K 12 DNA was included as a reference during each experiment.

Renaturation of DNA

The DNA samples were dialysed into the appropriate solvent and an aliquot of the dialysis solution was used for the absorbance reference. DNA was sheared by passage through a 27 gauge needle, using a 2 ml syringe and applying maximum pressure by hand. DNA solutions at concentrations of 6 to 20 $\mu\text{g/ml}$ were degassed by evacuation in a vacuum dessicator. The renaturation reaction was followed optically, with the DNA solutions contained in Spectrosil semi-micro cuvettes fitted with teflon stoppers and the absorbance at 260 nm being measured in a Gilford 2400 automatic recording spectrophotometer. In any experiment, two or three samples were run simultaneously.

In one experiment, after determining the OD_{260} of the native DNA, the DNA was denatured by adding one part 1.0 M NaOH to 8 parts of the DNA solution in 4.5 x SSC. After 10 minutes at room temperature the OD_{260} of

the denatured DNA was read. The solution was then neutralized by adding one part 2 M NaH_2PO_4 , mixed well, and absorbance readings begun immediately while the solution equilibrated to the renaturation temperature. DNA was renatured at $70 \pm 1.0^\circ\text{C}$. This is $(T_m - 28)^\circ$ for *E. coli* DNA and $(T_m - 22)^\circ$ for T4 DNA at this ionic strength (74). The T_m for *Acetabularia* chloroplast DNA has not been determined in a solvent of this ionic strength, but from the base compositions the T_m of the chloroplast DNA should be between those of *E. coli* DNA and T4 DNA.

In another experiment, DNA in SSC was melted as described above. The temperature was then lowered to $63 \pm 0.5^\circ$ and the DNA allowed to renature.

In other experiments, after determining the absorbance of the native DNA, the DNA in 0.1 x SSC was heated on a boiling water bath for 12 to 15 minutes. Using a warm pipette, 1.625 ml hot denatured DNA was added to 0.175 ml 20 x SSC (final concentration 2 x SSC) in a cuvette equilibrated to 65° . The solutions were mixed and absorbance readings begun immediately. Since the temperature recorded on the chart differed slightly from the actual temperature of the solutions in the cuvettes, an additional cuvette containing 2 x SSC was included. The temperature of this solution was monitored with a Tele-Thermometer (Yellow Springs Instrument Company) and used to calibrate the temperature recorded on the chart.

Absorbance values were corrected as necessary for changes in the volume of the DNA solution. Corrections for thermal expansion of the solvent were also made.

The chloroplast DNA solution, in particular, contained a contaminant of unknown nature which had a considerable absorbance at 260 nm. For this

reason, the concentration of DNA in each sample was determined by a micro assay using Burton's modification of the diphenylamine assay (15). The percentage of the OD₂₆₀ which was due to DNA was then calculated, and all absorbance readings corrected according to this value.

The following equations given by Wetmure and Davidson (74) were then used to determine the k_2 :

$$\frac{A_0 - A_\infty}{A - A_\infty} = \frac{k_2 P_T}{2} t + 1 \quad \text{and } P_T = 1.47 \times 10^{-4} A_\infty \text{ mole l}^{-1}$$

where A_∞ is the absorbance of native DNA;

A_0 is the maximum absorbance of denatured DNA;

A is the absorbance of the partially renatured DNA at time t ;

t is the time, in seconds,

P_T is the total DNA phosphate concentration;

and k_2 is the second order renaturation rate constant.

Since the rate of renaturation of the DNA is influenced by the ionic strength of the solvent, all k_2 values were normalized to a solvent monovalent cation concentration of 1.0 M, using values of relative reassociation rates given by Britten (11). Base composition of the DNA also affects its renaturation rate, therefore k_2 values for *Acetabularia* chloroplast DNA and T4 DNA were normalized to 50% G+C, using data given by Wetmur and Davidson (39, 74).

Determination of alkaline sedimentation coefficients

Band velocity sedimentation was used to determine the sedimentation coefficient of denatured DNA (63,69). Sedimentation was carried out at 28,000 or 30,000 rpm in the AN-D rotor of a Spinco Model E ultracentrifuge,

at a temperature of 22° to 25°. Photographs were taken at 8 min or 16 min intervals and the negatives scanned with a Joyce-Leobl microdensitometer.

About 25 μ l of a DNA solution in 0.1 x SSC ($OD_{260} \approx 0.4$) was used in the sample well of a 12 mm Kel-F band forming centerpiece. In some cases 0.5 ml of 0.9 M NaCl, 0.1 M NaOH was used as the bulk solution and the alkaline sedimentation coefficient ($S_{20,w}^{pH13}$) determined directly. In other cases, the DNA was heat denatured on a boiling water bath and quick cooled on ice before being loaded into the sample well. The bulk solution was 1.0 M NaCl, 0.05 M sodium citrate and the alkaline sedimentation coefficient was calculated from the following relationship derived from equations given by Studier (63):

$$\log S_{20,w}^{pH13} = \frac{0.400}{0.549} (\log S_{20,w}^{pH7, \text{denatured}} - \log 0.0105) + \log 0.0528$$

RESULTS

Attempts to obtain axenic cultures of *Acetabularia*

Washing *Acetabularia* cysts with seawater, alone, was important in reducing the amount of bacterial contamination. Plating of 0.1 ml of medium used for the first wash resulted in heavy, confluent white growth of bacteria. After the fifth wash, 0.1 ml of washing medium gave rise to 300-400 bacterial colonies. Treatment of cysts with SDS was very effective against bacteria which produced white colonies, but not as effective against the contaminants which gave rise to yellow colonies. Although axenic cultures did not result, the degree of contamination was slight, with 0.1 ml of the final washing medium giving rise to 5-10 bacterial colonies. Lysozyme was not as effective as SDS in eliminating the white contaminants, but was more effective against the yellow contaminants. Treatment of cysts with lysozyme followed by SDS was more effective than either of these agents alone, although completely axenic cultures still were not obtained. Antibiotic treatment was helpful in reducing the number of bacteria, but was not sufficient to produce axenic cultures.

Argyrol was the most effective agent for eliminating bacterial contaminants but it was also the most damaging to *Acetabularia* cysts. Cysts treated with argyrol after being treated with SDS failed to germinate, and died. When cysts were treated with argyrol alone, or when SDS treatment followed the treatment with argyrol, however, the cysts survived and germinated. This suggests that cysts which have been previously treated with SDS may be more permeable to argyrol and that argyrol is toxic to the cysts if it penetrates them. Some axenic cultures of *Acetabularia* were obtained

by treating the cysts with argyrol, but in most cases contamination was accidentally introduced into the cultures before the *Acetabularia* cells were fully grown. When *Acetabularia* cultures did remain axenic, the cells did not appear to grow as well as non-axenic cultures. Whether this was due to a lack of some metabolite which is supplied by the bacteria in contaminated cultures, or whether residual traces of Ag^{++} from the argyrol inhibited growth of the *Acetabularia* cells, was not investigated.

At one point, half of the stock cultures of *Acetabularia* became contaminated with *Nanachlores*. Probably the cysts were contaminated with *Nanachlores* before germination. It was observed that the *Acetabularia* cells arising from cysts which had been treated with SDS did not have any *Nanachlores* growing in with them, but all the cultures arising from *Acetabularia* cysts which had not been treated with SDS were contaminated with *Nanachlores*.

Bacterial contamination in chloroplast preparations

Several types of bacterial contaminants were present in the chloroplast preparations. These are listed in table I, together with the buoyant densities of their DNA. The first four types of bacteria listed in the table were the ones most commonly found in chloroplast preparations. Since the buoyant densities of DNA from these contaminants differ from the buoyant density of *Acetabularia* chloroplast DNA, it was possible to separate chloroplast DNA from bacterial DNA by means of a preparative CsCl gradient. Although this was done in preliminary experiments, the amount of bacterial contamination was low enough that this was not considered necessary with later preparations.

Table I: Bacterial contaminants of chloroplast preparations

Macroscopic Appearance	Microscopic Appearance	Gram Reaction	Buoyant Density
a) shiny, white, smooth, domed colony with entire margin	motile rod	negative	1.716 g/cm ³ 1.743 g/cm ³
b) creamy-white, shiny, domed colony with entire margin	motile; short, fat rods	positive	1.718 g/cm ³
c) small shiny, golden-yellow, flat colony. Colonies have "fried-egg" appearance on old plates	non-motile; long, thin rods	positive	1.699 g/cm ³
d) very small, shiny, golden-yellow colony	non-motile; long, filamentous rods, straight and curved; form networks. Networks broken up by lysozyme but cells not lysed	? negative	1.698 g/cm ³ 1.681 g/cm ³ (polysaccharide?)
e) shiny, yellow, flat colony with irregular margin; hydrolyzes agar	non-motile; long filamentous rods	positive	1.697 g/cm ³
f) shiny, creamy-white domed colony with irregular margin	non-motile rod occurring singly and in short chains	negative	1.715 g/cm ³
g) diffuse white, flat colony with irregular margin	non-motile rod occurring singly, paired and in short chains	negative	1.716 g/cm ³
h) shiny, orange, flat colony with entire margin	non-motile, long rods, some in chains curved (filaments?)	positive	1.706 g/cm ³

The degree of contamination in chloroplast preparations ranged from one bacterium per 6500 chloroplasts to one bacterium per 107,000 chloroplasts. If a chloroplast contains the same amount of DNA as a bacterium, the bacteria may have contributed 0.001% to 0.015% of the DNA in the final preparation. Assuming that a chloroplast contains one tenth the amount of DNA in a bacterium, at most the amount of bacterial DNA in the final preparation could be 0.15%.

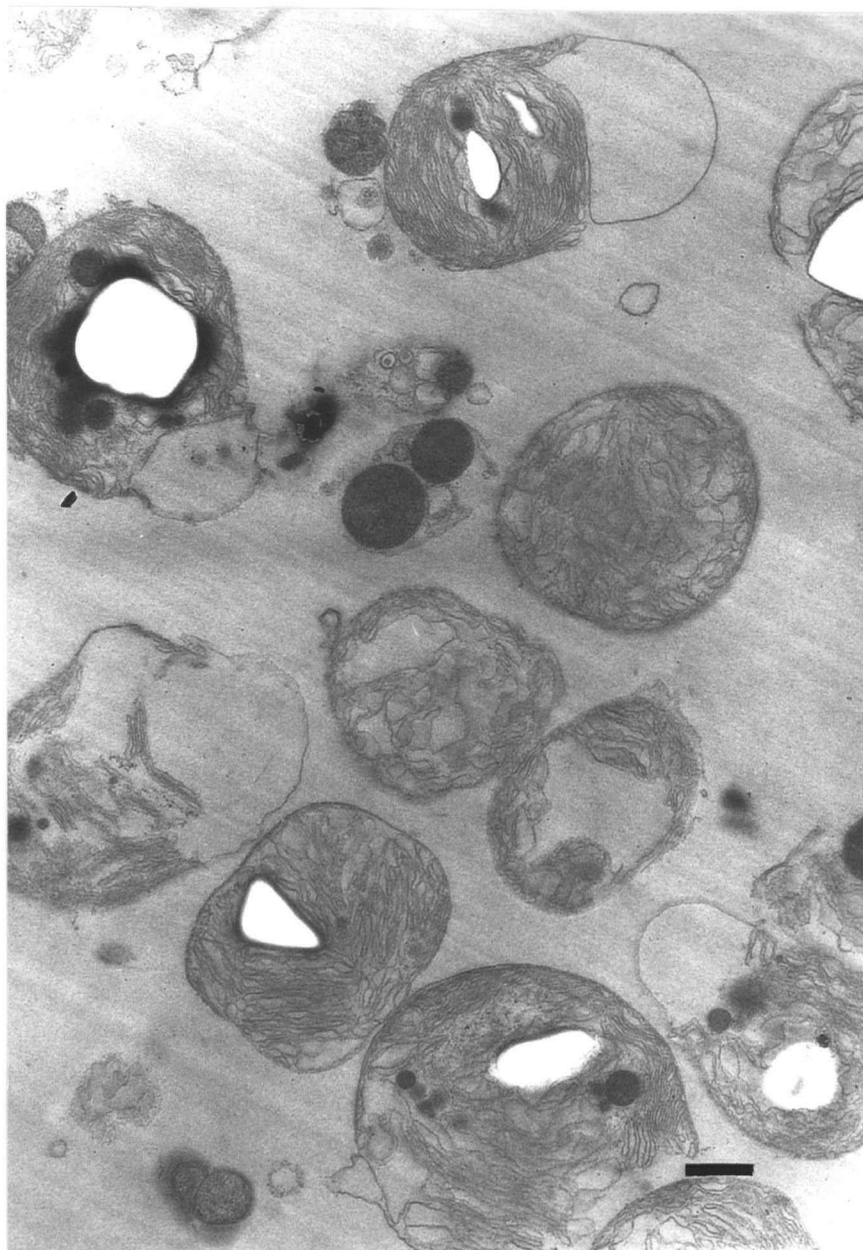
Electron Microscopy

Sections of isolated chloroplasts observed with the electron microscope showed mostly intact chloroplasts, although in many cases there was bulging of the membranes (fig. 1). This bulging may have been caused by osmotic changes during the fixation of the chloroplasts. No bacterial profiles were seen.

For chloroplasts which were sedimented through a buffered sucrose solution, one mitochondrial profile was seen for every 5 chloroplast profiles. Fewer mitochondria were observed when the chloroplasts were sedimented through A medium during the isolation procedure. In this case, one mitochondrial profile per ten chloroplast profiles was seen. For this reason, except for a few preliminary preparations, A medium was used rather than sucrose solution during the final steps of the chloroplast isolation.

Although only one mitochondrial profile was seen per 10 chloroplast profiles, the actual ratio of mitochondria to chloroplasts was probably much higher than this. Since a chloroplast is larger than a mitochondrion, the probability of sectioning through a chloroplast is greater than the probability of sectioning through a mitochondrion. The mean volume of an

Figure 1: Thin sections of *Acetabularia mediterranea* chloroplasts fixed in glutaraldehyde and post-fixed OsO_4 . Sections were stained with uranyl acetate followed by lead citrate. Bar = 1 μ (x 10,000.)



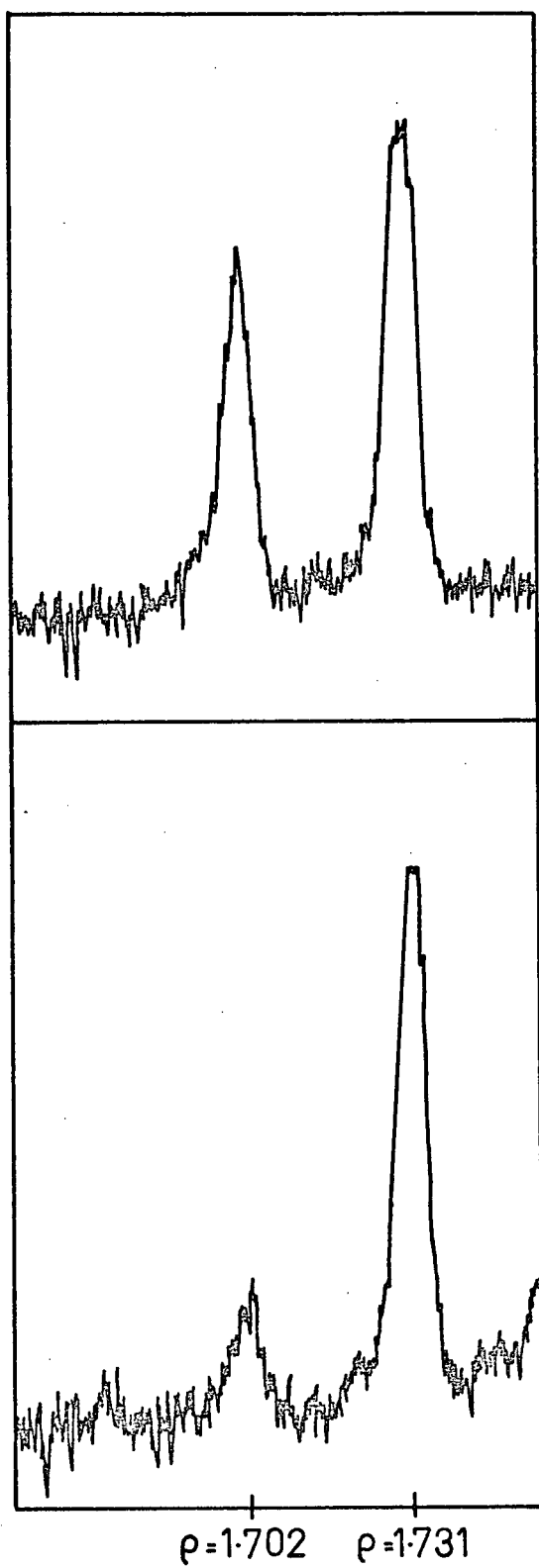
Acetabularia chloroplast is $8 \mu^3$ (55). Assuming an ellipsoid shape for a mitochondrion, and by measuring the diameters of mitochondrial profiles observed with the electron microscope, the mean volume of an *Acetabularia* mitochondrion is calculated to be approximately $0.4 \mu^3$. Assuming that an equal number of chloroplasts and mitochondria are randomly distributed in a given volume of embedding medium (this may not be a valid assumption), then the chances of sectioning through a chloroplast are 20 times greater than the chances of sectioning through a mitochondrion. Thus, the actual ratio could be as high as two mitochondria for every chloroplast.

The amount of DNA in an *Acetabularia* mitochondrion is not known, but it appears to be less than is in a chloroplast (75). From buoyant density studies of the cytoplasmic DNAs of *Acetabularia*, there appears to be about 10 times more chloroplast DNA than mitochondrial DNA (32). Assuming, then, that a mitochondrion contains one tenth the amount of DNA that is in a chloroplast, mitochondrial DNA could contribute as much as 20% of the DNA in the final preparation. In DNA from purified chloroplasts, however, there is no measurable amount of DNA at the buoyant density of mitochondrial DNA (fig. 2), $\rho = 1.714 \text{ g/cm}^3$ (32).

Buoyant density and T_m of *Acetabularia* chloroplast DNA

Acetabularia chloroplast DNA from two different preparations was centrifuged in an analytical CsCl density gradient. In both cases, a single peak with a buoyant density of 1.702 g/cm^3 was observed (fig. 2). This is different from the buoyant density of the DNA of any of the bacterial contaminants (table I). Using the relationship given by Schildkraut et al. (51), $\rho = 1.702 \text{ g/cm}^3$ corresponds to a base composition of 42.8% G+C. A

Figure 2: Densitometer tracings of ultraviolet photographs of *Acetabularia mediterranea* chloroplast DNA after analytical density-gradient centrifugation in CsCl. *Micrococcus lysodeikticus* DNA ($\rho = 1.731 \text{ g/cm}^3$) was used as density reference.



buoyant density of 1.702 g/cm^3 predicts a T_m of 87.0°C in SSC and 71.2° in $0.1 \times \text{SSC}$, using the following equations given by Mandel et al (41):

$$T_m (\text{SSC}) = 418.2 (\rho - 1.494)$$

$$T_m (\text{SSC}/10) = 512.2 (\rho - 1.563)$$

In one attempt to determine the T_m in SSC, the curve began to level after a hyperchromic increase of about 31%, but this was followed by a sudden hyperchromic rise above 95° (fig. 3). This sudden increase in hyperchromicity may have been due to evaporation of the solvent, since about 1.5 ml of sample was contained in a cuvette which has a capacity of 1.8 ml. Another possible explanation is that this sudden increase was an artifact produced by a gas bubble. Taking 31.5% as the maximum hyperchromicity, the T_m in SSC of the *Acetabularia* chloroplast DNA is 86.7° , based on a $T_m = 90.5^\circ$ for *E. coli* K 12 DNA (41). This is in good agreement with the predicted value of 87.0° .

Acetabularia chloroplast DNA had 22.8% hyperchromicity in $0.1 \times \text{SSC}$, with a T_m of 70.7° (fig. 3) based on a $T_m = 75.7^\circ$ for *E. coli* K 12 DNA (41). This is 0.6° lower than predicted, but this may be due to the low molecular weight of the DNA (20,21). The melting transition was not smooth. This, also, is possibly due to the fact that this DNA was sheared to a low molecular weight (about 7.5×10^5 daltons). If there is a high degree of intramolecular heterogeneity in *Acetabularia* chloroplast DNA, then shearing of the DNA could produce fragments of varying base compositions. A differential plot of the thermal transition of *Acetabularia* chloroplast DNA shows multiple peaks (fig. 4), which is also indicative of intramolecular heterogeneity (23).

Figure 3: Melting curves of native DNA. (...) *Acetabularia mediterranea* chloroplast DNA melted in 0.1 x SSC; (ooo) *Acetabularia mediterranea* chloroplast DNA melted in SSC; (Δ — Δ) *E. coli* DNA melted in SSC.

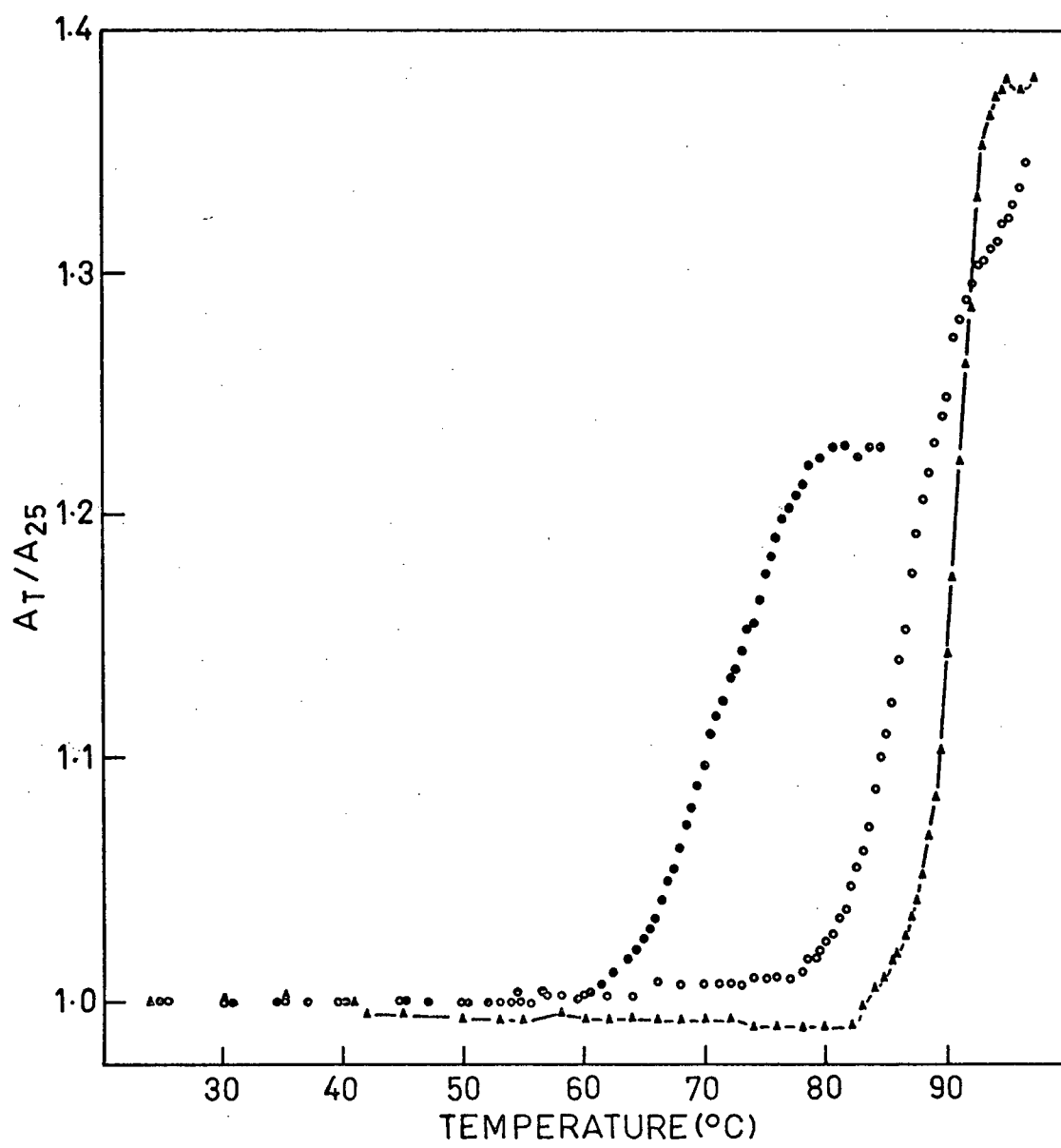
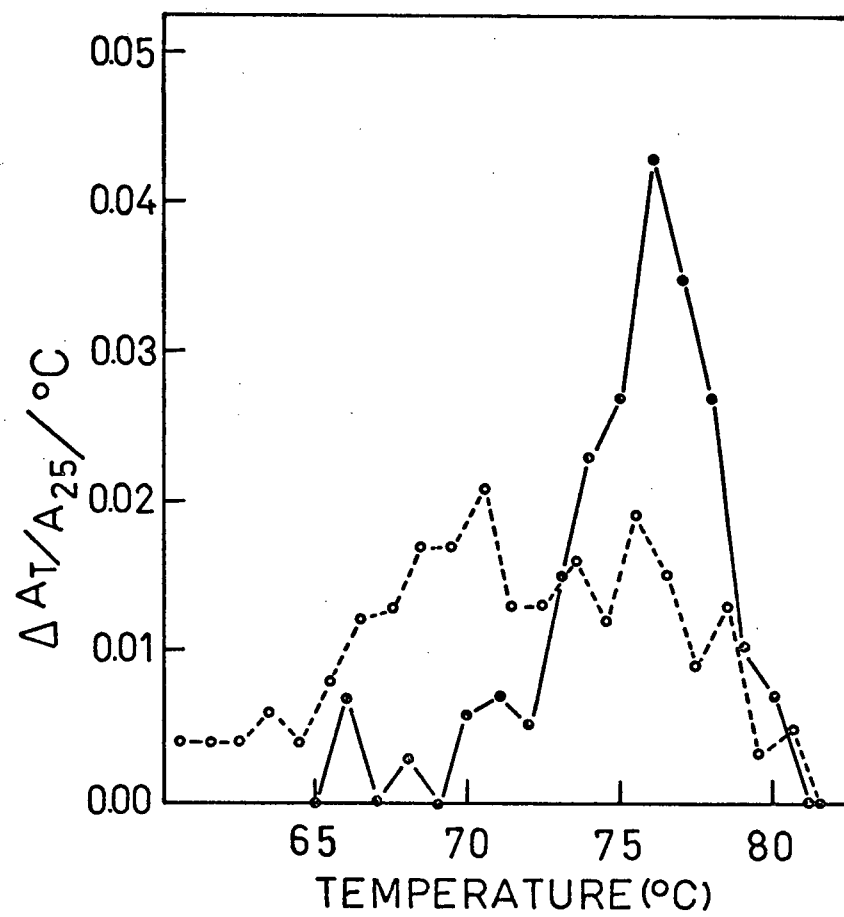


Figure 4: Differential melting plot of native DNA in 0.1 x SSC
(o--o) *Acetabularia mediterranea* chloroplast DNA;
(.=-.) *E. coli* DNA.



Acetabularia chloroplast DNA shows a very broad transition width for melting. The transition width ($^{\circ}\text{C}$ between 17% and 83% of total hyperchromicity) of melting in SSC is 8.2° and in $0.1 \times \text{SSC}$ is 9.7° . This could be partly due to the low molecular weight of the DNA (20,21), but may also be indicative of a high degree of intramolecular heterogeneity (42).

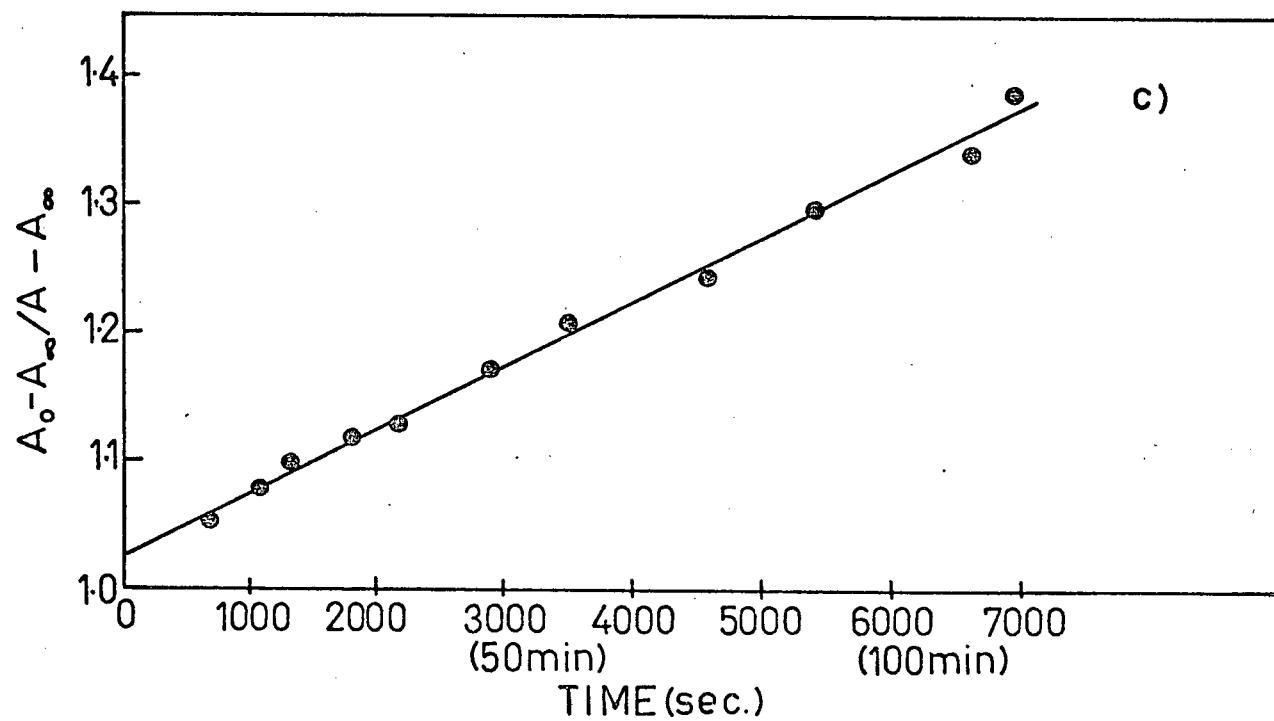
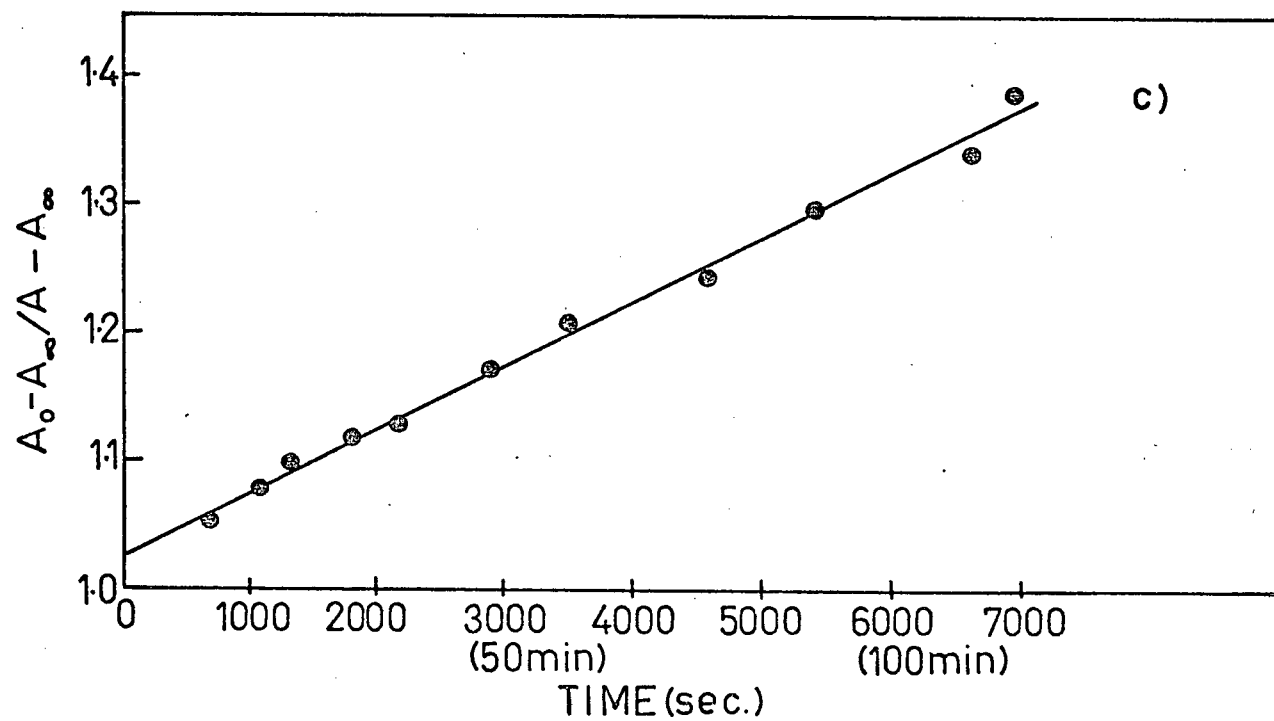
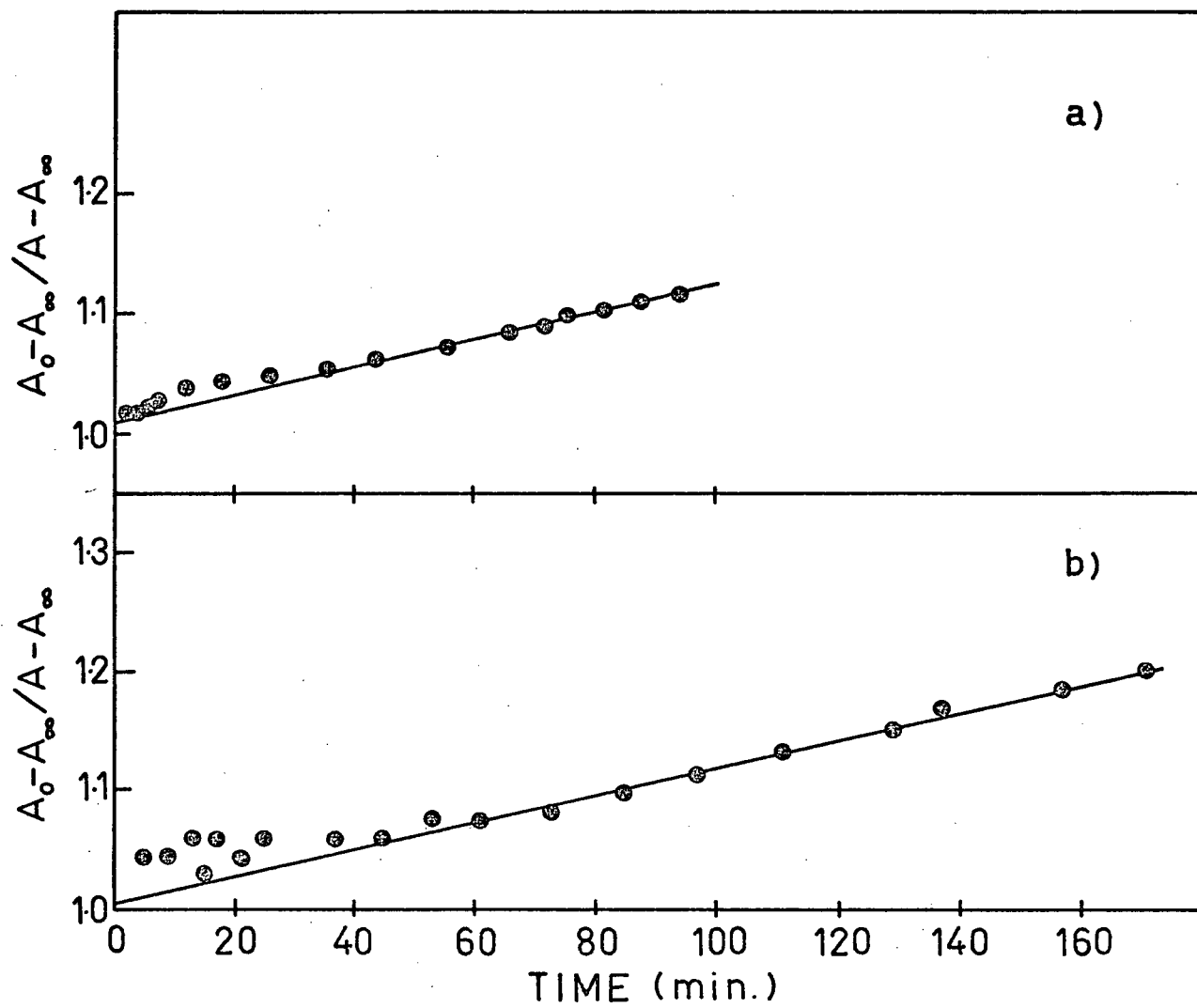
Renaturation and kinetic complexity of *Acetabularia* chloroplast DNA

The rate plots for the renaturation of *Acetabularia* chloroplast DNA are given in figure 5. There appears to be a small amount of a fast renaturing component, representing about 3% of the total DNA. This is probably due to mitochondrial DNA. In one experiment (fig. 5b) there was a considerable lag before the reaction proceeded. This is difficult to explain, but may be an artifact due to insufficient mixing of the DNA solution in $0.1 \times \text{SSC}$ with the concentrated ($20\times$) SSC solution. Also, the concentration of DNA in this sample was very low (about $6 \mu\text{g/ml}$), and this may be part of the reason for this lag.

In the first renaturation experiment, the DNA was alkali denatured. No hyperchromicity of the *Acetabularia* chloroplast DNA was observed. When the neutralized sample was subsequently run in an analytical CsCl density gradient, no band was detected, suggesting that the DNA had been degraded. It is very unlikely that this alkali lability is due to the presence of unusual bases in *Acetabularia* chloroplast DNA, since the melting behavior of the chloroplast DNA and its buoyant density in CsCl correlate well (41,42). The problem of alkali lability has been encountered with mitochondrial DNA (10). It is probable that free radicals are generated during the DNA

Figure 5: Renaturation rate plots for *Acetabularia mediterranea* chloroplast DNA.

- (a) Experiment 2: DNA concentration approximately 15 $\mu\text{g/ml}$;
renatured in SSC at $63 \pm 0.5^\circ$; $k_2 = 5.90 \text{ l mole}^{-1} \text{ sec}^{-1}$
 - (b) Experiment 3: DNA concentration approximately 6 $\mu\text{g/ml}$;
renatured in 2 x SSC at $65 \pm 0.5^\circ$; $k_2 = 5.00 \text{ l mole}^{-1} \text{ sec}^{-1}$.
 - (c) Experiment 4: DNA concentration approximately 11 $\mu\text{g/ml}$;
renatured in 2 x SSC at $65 \pm 0.5^\circ$; $k_2 = 7.62 \text{ l mole}^{-1} \text{ sec}^{-1}$
- k_2 values corrected for ionic strength of solvent and normalized to 50% G+C



isolation procedure (10) and these attack the N-glycosidic bond (49). This results in base elimination and subsequent chain breakage at $\text{pH} > 7$ (49). Therefore, in subsequent experiments, heat denaturation was used rather than alkaline denaturation.

In experiment 4, after the DNA had been renatured for 34 hours (*E. coli* DNA 88% renatured; *Acetabularia* chloroplast DNA 59% renatured) it was quickly cooled and dialysed into 0.1 x SSC in the cold. Following this, the DNA was melted. There was some loss of O.D. for the chloroplast DNA sample, even after correcting for the volume change due to dialysis. It is probable that some degradation of the DNA occurred during the renaturation reaction and subsequently small fragments were lost during dialysis. The melting curves for the renatured DNA samples are shown in figure 6. The renatured *Acetabularia* chloroplast DNA has a T_m of 70.0° , which is 0.7° lower than the T_m of native chloroplast DNA. Renatured *E. coli* DNA also has a T_m which is lowered by 0.7° . This lowering of the T_m indicates about 1% mismatched base pairs in the renatured DNA (40).

The renaturation rate constants, alkaline sedimentation coefficients and kinetic complexities of a number of DNA samples are presented in table II. The kinetic complexity of *E. coli* DNA should be 2.5×10^9 daltons, in agreement with its analytical complexity (74). The values obtained in some of these experiments, however, are lower than this. The most probable explanation is that the values for the alkaline sedimentation coefficients are in error. Sedimentation coefficients were determined after the DNA had been used for the renaturation reaction. After this prolonged exposure to a high temperature there would have been some degradation of the DNA (22), resulting

Figure 6: Thermal transition profiles of native and renatured DNA melted in 0.1 x SSC. (a) *Acetabularia mediterranea* chloroplast DNA; (...) native DNA, $T_m = 70.7^{\circ}$; (ooo) renatured DNA, $T_m = 70.0^{\circ}$. (b) *E. coli* DNA; (...) native DNA, $T_m = 75.7^{\circ}$; (ooo) renatured DNA, $T_m = 75.0^{\circ}$.

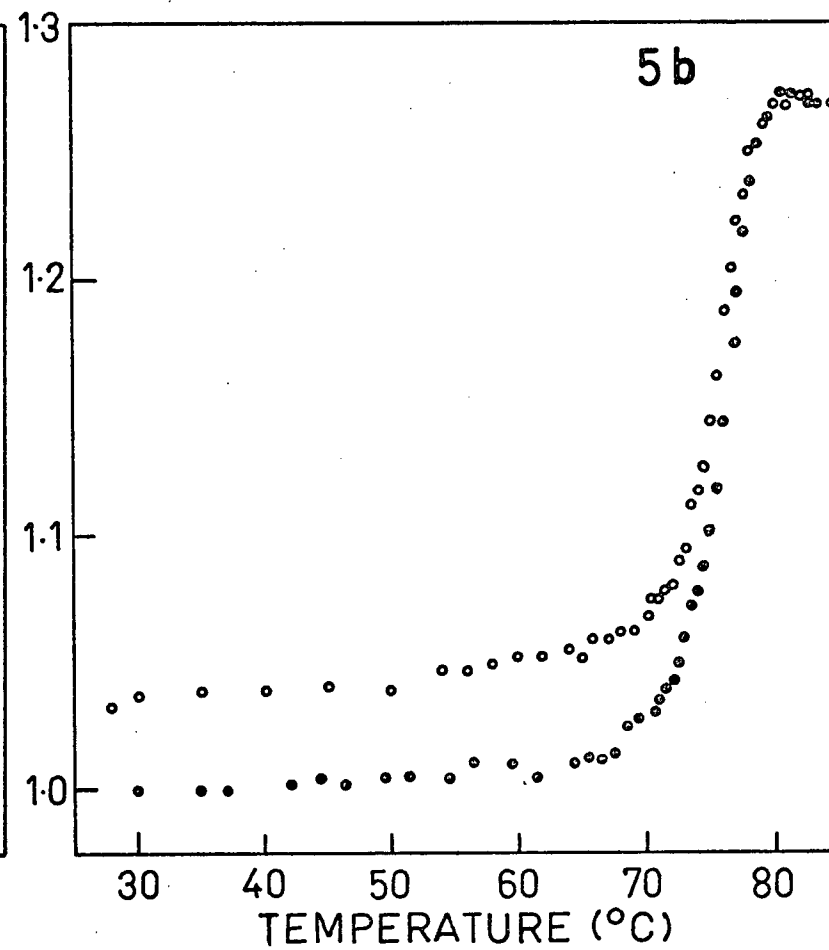
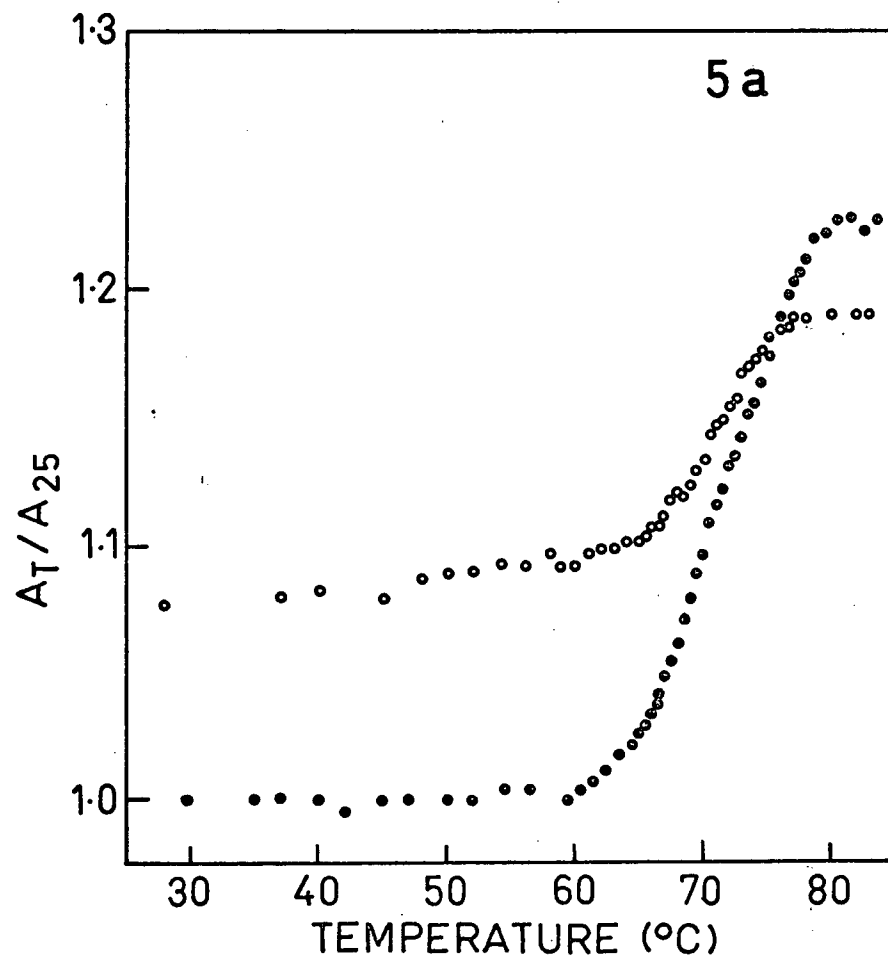


Table II. Kinetic complexities of DNA from different sources

Exp. No.	Source of DNA	P_T (mole l^{-1})	k_2 (1 mole $^{-1}$ sec $^{-1}$)	$S_{20,w}^{pH13}$		Kinetic Complexity (daltons)
				meas. before	meas. after	calculated *
1	bacteriophage T4	3.01×10^{-5}	197		24	1.5×10^8
1	<i>E. coli</i>	3.57×10^{-5}	11.60		14.9	1.4×10^9 2.5×10^9
2	<i>E. coli</i>	5.73×10^{-5}	13.57		13	1.0×10^9 2.6×10^9
33	<i>E. coli</i>	3.92×10^{-5}	5.31	28	6	1.0×10^9 2.5×10^9
4	<i>E. coli</i>	4.54×10^{-5}	6.88		10	1.4×10^9 2.5×10^9
5	<i>E. coli</i>	6.00×10^{-5}	6.05	13.4		2.3×10^9
2	<i>Acetabularia mediterranea</i> chloroplasts	4.39×10^{-5}	5.90		6	8.7×10^8 2.3×10^9
3	<i>Acetabularia</i> chloroplasts	1.82×10^{-5}	5.00		9	1.7×10^9 4.5×10^9
4	<i>Acetabularia</i> chloroplasts	3.10×10^{-5}	7.62		7	8.1×10^8 1.4×10^9

* $S_{20,w}^{pH13}$ corrected to obtain a kinetic complexity of 2.5×10^9 daltons for *E. coli* DNA

in lower values for the sedimentation coefficients. In one experiment with *E. coli* DNA, an aliquot of DNA for determination of the sedimentation coefficient was taken at the start of the experiment, immediately after the DNA had been heat denatured. In this case, a value of 2.3×10^9 daltons was obtained for the kinetic complexity (table II, experiment 5). In another experiment (table II, experiment 2), a value of $S_{20,w}^{pH13} = 13$ was determined on a sample of DNA after it had been used in the renaturation reaction. With another sample of sheared DNA from the same batch, but which had not been heat denatured and renatured, $S_{20,w}^{pH13} = 28$. Using 13 as the alkaline sedimentation coefficient, a kinetic complexity of 1.0×10^9 daltons is calculated for *E. coli* DNA, but using $S_{20,w}^{pH13} = 28$, the kinetic complexity of *E. coli* DNA is 2.6×10^9 daltons. Therefore, the values of the sedimentation coefficients were corrected to obtain a kinetic complexity of 2.5×10^9 daltons for *E. coli* DNA. A corresponding correction was made for the sedimentation coefficients of the *Acetabularia* chloroplast DNA. This correction assumes that the chloroplast DNA was degraded to the same degree as the *E. coli* DNA. This may not be a valid assumption. Indeed, a look at the corrected values given in table II would suggest that this assumption is not valid. One would expect the DNA in experiment 4 to be the most degraded, since it was exposed to a high temperature for the longest period of time, yet it has the smallest correction. On the other hand, the correction applied in experiment 3 is probably too large. An attempt was made to determine a correction factor for the alkaline sedimentation coefficients by shearing bacterial DNA under the same conditions as had been used to shear the chloroplast DNA (see below).

The kinetic complexity of *Acetabularia mediterranea* chloroplast DNA is at least 8.1×10^8 daltons, and is probably greater than this. The average result of three separate experiments gives a kinetic complexity of $1.1 \pm 0.4 \times 10^9$ daltons. This agrees closely with an analytical complexity of 1.4×10^9 daltons (2.3×10^{-15} g) of DNA as determined by Green (Unpublished). Thus, it appears probable that the chloroplast DNA exists as one unique sequence, with no repeated segments. At most, there could be two copies of the unique sequence.

Sedimentation coefficients

If the method of shearing produced the same size of DNA fragments fairly reproducibly, then any DNA sample could be taken and sheared under the same conditions as were used for shearing the DNA in the renaturation experiments. The alkaline sedimentation coefficients could then be determined for these samples and the values obtained could be used in calculating the kinetic complexities. This was done, using purified DNA, which was extracted from one of the bacterial contaminants. Solutions of the DNA were adjusted to the concentrations used in the renaturation experiments. Three aliquots of each concentration of DNA were taken and each aliquot sheared once or twice with a 27 gauge needle, depending on the conditions used previously. The DNA samples were concentrated with Aquacide II, dialyzed into 0.1 x SSC, and then the alkaline sedimentation coefficients were determined. The results of these experiments are presented in table III.

It can be seen that within the concentration range used, the concentration of DNA had little effect on the size of the DNA fragments produced

Table III. Alkaline sedimentation coefficients of sheared DNA Samples

	DNA concentration ($\mu\text{g/ml}$)				
	6.5	8	10	12	15
sheared:	x2	x1	x2	x1	x1
$S_{20,w}^{\text{pH13}}$	25.6	25.8	26.2	26.6	25.4
	23.8	24.2	25.3	26.4	27.2
	25.0	31.7	26.6	29.2	25.5
mean:	23.7 ± 1.12	27.2 ± 3.92	26 ± 0.21	27.4 ± 1.56	26.0 ± 1.01

by shearing with a 27 gauge needle. There was some variation in the sedimentation coefficients obtained for different DNA samples at the same concentration, particularly when the DNA was sheared only once. These differences in $S_{20,w}^{\text{pH13}}$ for different samples of DNA can lead to considerable variation in the calculated value of the kinetic complexity, as shown in table IV using the k_2 determined for *E. coli* DNA in experiment one. In each case, the value for the kinetic complexity is too high. The sedimentation coefficients determined in these experiments are generally higher than the values measured for the DNA samples used in the renaturation experiments. This gives support to the idea that some DNA degradation occurred during the renaturation experiments. On the other hand, both the *E. coli* DNA and the chloroplast DNA (particularly the chloroplast DNA) used for the renaturation experiments were handled more extensively than this

Table IV. Variation in kinetic complexity with different values of $S_{20,w}^{pH13}$

Source of DNA	DNA concentration	k_2	$S_{20,w}^{pH13}$	kinetic complexity (daltons)
<i>E. coli</i> *	12 μ g/ml	11.6 l mole ⁻¹ sec ⁻¹	14.9	1.4×10^9
			24.0	2.5×10^9
<i>E. coli</i>	12 μ g/ml	11.6 l mole ⁻¹ sec ⁻¹	26.4 [†]	2.8×10^9
			27.4 [†]	2.9×10^9
			29.2 [†]	3.2×10^9

* data taken from experiment 1.

† taken from table III.

bacterial DNA and, therefore, possibly had a lower molecular weight before shearing with the needle. Since the sedimentation coefficients determined in these experiments give kinetic complexities for *E. coli* DNA which are too large, it was not possible to use a correction factor based on them.

DISCUSSION

Acetabularia mediterranea chloroplast DNA has a buoyant density in CsCl of 1.702 g/cm^3 . This agrees quite closely to the value of 1.704 g/cm^3 reported by Green et al. (32) and Heilporn and Limbosch (34), and indicates a based composition of 42.8% G+C. The T_m of *Acetabularia* chloroplast DNA in SSC is 86.7° . This gives a value of 43% G+C for the base composition, as calculated from equation 5 of Mandel et al. (41) using *E. coli* K 12 DNA as the standard. The close agreement of the base compositions calculated from the buoyant density and the melting temperature indicates the absence of any unusual bases in *Acetabularia mediterranea* chloroplast DNA (36, 41, 42). Chromatographic data of Gibor supports this conclusion. *Acetabularia* chloroplast DNA which was hydrolyzed enzymatically and then chromatographed showed only deoxyadenosine, thymidine, deoxycytosine and deoxyguanosine (26).

Acetabularia mediterranea chloroplast DNA melts with a T_m of 70.7° in $0.1 \times \text{SSC}$. This is 0.6° lower than expected for a DNA of 43% G+C content. This lowering of the T_m is probably due to the low molecular weight of the DNA (20,21). The melting transition of *Acetabularia* chloroplast DNA is very broad. This is partly due to the low molecular weight of the DNA (20,21) but could also be indicative of a high degree of intramolecular heterogeneity (42). The observed unevenness of the melting transition, especially noticeable when the DNA was melted in $0.1 \times \text{SSC}$, may also be indicative of a high degree of intramolecular heterogeneity. This uneven melting transition is similar to that reported for the mitochondrial DNA of *Drosophila melanogaster* (47). Polan et al. (47) attribute

the multitransitional behavior of *D. melanogaster* mitochondrial DNA to differences in base composition in various portions of the molecule. A differential plot of the melting transition of *A. mediterranea* chloroplast DNA shows multiple peaks, which supports the conclusion that this DNA has a high degree of intramolecular heterogeneity. The melting curves of chloroplast DNAs from *Chlorella pyrenoidosa* (6), *Chlamydomonas reinhardtii* (5,72) and *Euglena gracilis* (65) show considerable intramolecular heterogeneity in these DNAs as well.

The kinetic complexity of *Acetabularia* chloroplast DNA, based on the average value obtained in three separate experiments is $1.1 \pm 0.4 \times 10^9$ daltons, with the least value being 8.1×10^8 daltons (see table II). *E. coli* DNA which was renatured at the same time as the chloroplast DNA, under the same conditions, has a kinetic complexity lower than the literature value of 2.5×10^9 daltons (74). This is probably due to errors in the values of the alkaline sedimentation coefficients (see Results). Therefore, the kinetic complexity of *Acetabularia mediterranea* chloroplast DNA is probably greater than 1.1×10^9 daltons. This would indicate that the amount of DNA per chloroplast is more than the 1×10^{-16} g (6×10^7 daltons) estimated by Gibor and Izawa (27). If the analytical complexity of 1.4×10^9 daltons (2.3×10^{-15} g) determined by Green (unpublished) is correct, then at most there could be two copies of the DNA nucleotide sequence, based on a kinetic complexity of 8.1×10^8 daltons. If a fraction of the DNA is highly repetitious, this would have been evident as a separate kinetic component in the renaturation reaction (12, 13, 14, 74). A very small amount (approximately 3% of the total DNA) of a faster renaturing component was observed but this is most likely due to mitochondrial

DNA. Since 8.1×10^8 daltons represents a minimum value, and the average value for the kinetic complexity ($1.1 \pm 0.4 \times 10^9$ daltons) is probably too low due to errors in the values of the sedimentation coefficients, it seems likely that there is no nucleotide sequence repetition in *Acetabularia mediterranea* chloroplast DNA. On the other hand, since 2.3×10^{-15} g represents an average amount of DNA per chloroplast, it is possible that while some chloroplasts contain one copy of the genome, others may contain two or more copies. Other chloroplasts may not contain DNA if, during some chloroplast divisions, all of the DNA goes to one daughter chloroplast and none to the other. This would be compatible with the observations of Woodcock and Bogorad (75) that no DNA could be detected in 65-80% of chloroplasts, using four different methods; and that in those chloroplasts with detectable DNA the amount present was variable.

The kinetic complexities of chloroplast DNAs from a number of sources are shown in table V. The kinetic complexity of *Acetabularia mediterranea* chloroplast DNA is larger than the complexities of other chloroplast DNAs which have been investigated by renaturation kinetics. The larger kinetic component of kidney bean chloroplast DNA is the only value that approaches the kinetic complexity of *Acetabularia* chloroplast DNA. Crandall's results have never been published, however, and there is some question as to the purity of the DNA used in his experiments (M.D. Chilton, personal communication to B.R. Green). This throws doubt on the validity of his high value for the kinetic complexity of kidney bean chloroplast DNA. Thus, the kinetic complexity of *Acetabularia mediterranea* chloroplast DNA seems unusually large--but then, *Acetabularia* is an unusual organism. The fact that *Acetabularia* can survive for long periods following enucleation makes one

Table V: Kinetic complexities of chloroplast DNAs from various sources

Organism	Chloroplast DNA		Reference
	kinetic complexity (daltons)	Analytical complexity (daltons)	
<i>Acetabularia mediterranea</i>	$1.1 \pm 0.4 \times 10^9$	1.4×10^9	
<i>Euglena gracilis</i>	1.8×10^8		Stutz (64)
<i>Chlamydomonas reinhardtii</i>	1.94×10^8 $1-10 \times 10^6$ 2×10^8	5.16×10^9	Bastia <u>et al.</u> (5) Wells and Sager (72)
<i>Chlorella pyrenoidosa</i>	3×10^7 1.3×10^8		Bayen and Rode (6)
<i>Phaseolus vulgaris</i> (kidney bean)	7.4×10^6 7.5×10^8	7.2×10^8	Crandall (19)
Lettuce	3×10^6 1.2×10^8	2×10^9	Wells and Birnstiel (71)
Pea	9.5×10^7		Kolodner and Tewari (38)
Tobacco	1.14×10^8		Tewari and Wildman (67)

suspect that this ability may be related to the amount of genetic information in the chloroplasts. It hardly seems surprising that the chloroplast DNA of a uninucleate cell as large as *Acetabularia* has a greater unique sequence length than the few chloroplast DNAs of other organisms which have been studied to date.

Proponents of the symbiotic theory of the origin of chloroplasts suggest that there was a reduction in the amount of genetic information in the chloroplast as this information was transferred from the organelle-symbiont to the nuclear genome during the course of time, and the chloroplast became an integral part of the cell (61). If this is the case, then perhaps *Acetabularia* chloroplasts have not become integrated into the cell to the same degree as chloroplasts of other plants that have been studied. Bayan and Rode (6) suggest that since most chloroplast DNAs studied are quite similar in terms of kinetic complexity and heterogeneity, this is in agreement with the theory that chloroplasts of different organisms might have had a common origin. It is possible, however, that chloroplasts arose more than once, rather than as a result of a single evolutionary event.

Two kinetic components have been observed for the chloroplast DNA from some sources (6, 19, 71, 72), but for *Acetabularia mediterranea* chloroplast DNA there is only one component. A very minor, faster renaturing component was observed, but this probably results from contamination by mitochondrial DNA. It is interesting to note that with *Chlamydomonas reinhardtii* chloroplast DNA, Wells and Sager (72) observe two kinetic components, but Bastia et al. (5) report only the slower renaturing component. Perhaps this difference reflects differences in techniques used for isolating the DNA and carrying out the renaturation reaction.

The kinetic complexity of *Acetabularia mediterranea* chloroplast DNA ($1.1 \pm 0.4 \times 10^9$ daltons) is comparable to the kinetic complexities of DNAs from a number of free-living micro-organisms, such as *Hemophilus influenzae* ($1.01 \pm 0.18 \times 10^9$ daltons), *H. aegyptius* ($1.17 \pm 0.11 \times 10^9$ daltons),

Pasteurella multocida ($1.13 \pm 0.16 \times 10^9$ daltons), *Staphylococcus albus* ($1.12 \pm 0.02 \times 10^9$ daltons), *Neisseria catarrhalis* ($1.04 \pm 0.16 \times 10^9$ daltons) (3) and *Acholeplasma laidlawii* (1.10×10^9 daltons) (2). A genome size of 1.1×10^9 daltons represents 3.3×10^6 nucleotides, or 1.7×10^6 nucleotide pairs. This amount of DNA could code for a considerable number of proteins, in addition to coding for ribosomal RNA and transfer RNA.

Experimental evidence indicates that chloroplast ribosomal RNA and possibly some transfer RNAs are synthesized in the chloroplasts of *A. mediterranea* and *A. cliftonii* (8, 24, 25, 53). Incorporation of precursors into RNA by isolated chloroplasts is inhibited by darkness, actinomycin and deoxyribonuclease (8), indicating that this RNA is transcribed from chloroplast DNA. Farber's studies (24,25) of RNA metabolism in whole cells and cell fragments of *Acetabularia mediterranea* support the findings of Berger (8). The ribosomal RNA fractions found in *Acetabularia* together represent a chain of about 5×10^3 ribonucleotides (52). These would be coded by 5×10^3 deoxyribonucleotide pairs.

At least 1.6×10^3 DNA base pairs are required to code for transfer RNA, assuming 20 different species of chloroplast tRNA each with 80 nucleotides. This leaves 1,693,400 deoxyribonucleotide pairs (or less if the ribosomal RNAs and transfer RNAs are formed from larger precursor molecules) for regulatory and structural genes. The amount of DNA which is involved in the regulation of gene activity is not known. If this is ignored, then an approximate maximum number of proteins which can be coded by this amount of DNA can be estimated. If the ribosomal proteins are coded by the chloroplast DNA (although recent evidence suggests that some

of them may be under nuclear control--see below), they may account for approximately 1.1×10^4 amino acids (52) or 3.3×10^4 nucleotide pairs. This leaves 16.6×10^5 deoxyribonucleotide pairs, which could code for approximately 5.5×10^5 amino acids. Assuming an average protein chain length of 300 amino acids (70), then the chloroplast DNA might code for as many as 1800 proteins.

Theoretically, then, the chloroplast DNA could code for most, if not all, of the chloroplast proteins. What is the experimental evidence to suggest which proteins, if any, are coded by the chloroplast DNA?

Goffeau and Brachet (29) studied the incorporation of radioactive amino acids into proteins by chloroplasts isolated from anucleate fragments of *Acetabularia*. From the effects of a number of inhibitors on this process, these researchers concluded that the synthesis of chloroplast proteins is dependent on chloroplast DNA. Further studies showed that 60% of the total radio-activity is associated with the chloroplast membranes following incorporation of radioactive amino acids into proteins by isolated chloroplasts (28).

Using autoradiography, Shephard (56) compared the effects of enucleation and actinomycin D on the incorporation of nucleic acid and protein precursors by *Acetabularia* chloroplasts. He found that thymidine was still incorporated into chloroplast DNA one week after enucleation. Periods up to 5 weeks after enucleation had little effect on the ability of the chloroplasts to incorporate uridine into ribonuclease removable material. Treatment with actinomycin, however, resulted in the almost complete absence of labelled uridine from the plastid RNA of normal as well as anucleate cells. Incorporation of leucine by the chloroplasts was affected

very little by enucleation, but treatment with actinomycin greatly reduced leucine incorporation in normal and anucleate plants. Shephard concluded that replication of the chloroplast DNA is independent of the nucleus. He also suggested the evidence is strong that synthesis of plastid RNA and protein is under the direction of plastid DNA.

Craig and Gibor (18) found that photosynthetic activity decreased in whole and anucleate *Acetabularia* cells which had been maintained in the dark. Upon reillumination, however, both whole and anucleate cells regained photosynthetic activity. Treatment with puromycin totally inhibited recovery of photosynthetic activity, indicating that protein synthesis is necessary for this recovery. During treatment with actinomycin D, some recovery of activity was observed during the early stages of reillumination but full reattainment of photosynthetic activity did not occur in whole or anucleate cells. This suggests that transcription of chloroplast DNA is required during the light induced recovery of photosynthetic activity.

These conclusions conflict with evidence presented by Schweiger and others which demonstrates nuclear control over some of the chloroplast proteins. Schweiger et al. (54) studied the isozyme patterns of malic dehydrogenase by acrylamide gel disc electrophoresis, and found that they were species specific in four species of Dasycladaceae (*Acetabularia*). Following enucleation, the positions and relative concentrations of the different isozyme bands remained constant. Interspecific nuclear transplants, however, changed the isozyme patterns of the recipient species to that of the nucleus donor species. Thus, it appears that malic dehydrogenase, an enzyme associated with the chloroplasts, is controlled by the nucleus.

In a similar set of experiments, Reuter and Schweiger (50) demonstrated that lactic dehydrogenase, also associated with the chloroplasts, is coded by the cell nucleus. Malic dehydrogenase and lactic dehydrogenase are not enzymes which one expects to find associated with chloroplasts, but rather with mitochondria. It is quite possible that the chloroplast preparations used in these experiments were contaminated with mitochondria. (53).

Also using electrophoresis, Apel and Schweiger (1) studied the patterns of a membrane protein fraction from *A. calyculus* and *A. mediterranea* chloroplasts. The protein patterns were composed of 8 peaks common for both species and 3 peaks which were species specific. Six weeks after interspecific nuclear transplantation or implantation the protein pattern changed to that of the nucleus donor species. Thus, the species specific chloroplast proteins are nucleus dependent and appear to be coded by the nuclear DNA. This does not rule out the possibility that the peaks which are common to both species are due to proteins which are coded by chloroplast DNA. Another possibility is that the proteins which are coded by the nucleus constitute outer membrane proteins, while the inner chloroplast membrane components are coded by chloroplast DNA. The techniques used would not separate inner chloroplast membranes and outer chloroplast membranes. The incorporation of radioactive amino acids into the membrane protein fraction of *Acetabularia mediterranea* was also studied by these researchers. The electrophoretic pattern of the labelled chloroplast proteins consisted of at least 3 peaks. Both cycloheximide and chloramphenicol affected the incorporation of radioactivity into peaks 1 and 2, while

cycloheximide inhibited the incorporation into peak 3. Isolated chloroplasts incorporated amino acids only into the first two peaks. This indicates that some of the proteins in peaks 1 and 2 are probably synthesized on chloroplast ribosomes whereas peak 3 proteins are synthesized on cytoplasmic ribosomes.

More recently, Kloppstech and Schweiger (37) have used the same techniques to demonstrate species specific differences in the electrophoretic patterns of chloroplast ribosomal proteins from *A. mediterranea*, *A. cliftonii* and *A. crenulata*. Again, following interspecific nuclear implants and transplants, the protein patterns of the host species change to those of the nucleus donor species. The results indicate that those chloroplast ribosomal proteins which are species-specific are coded by the nuclear genome. They do not rule out the possibility that the protein bands which are the same in the electrophoretic patterns for all 3 species may be coded by chloroplast DNA.

Ceron and Johnson (16) have studied the control of protein synthesis during the development of *Acetabularia*. Using zone electrophoresis, they analyzed proteins from the soluble, chloroplastic and cell membrane fractions of axenically grown *Acetabularia crenulata*. Cells from various developmental stages were labelled with ^{14}C leucine. Throughout development, the electrophoretic patterns of proteins from the soluble fraction remained constant with respect to the number of bands, but the relative rates of synthesis of several proteins changed. Proteins from the membrane fraction, however, showed changes in both the number of bands in the electrophoretic pattern and in the relative synthetic rates of various protein species. After the cells were enucleated, there was an initial

decrease in the overall rate of synthesis of proteins from the soluble fraction. This returned to normal levels by 6 weeks post enucleation. Enucleation did not affect the electrophoretic pattern of membrane fraction proteins. Analysis of soluble proteins from purified chloroplasts after their labelling in normal and anucleate cells, showed that both the staining and autoradiographic patterns were essentially unchanged even 4 weeks after enucleation. When isolated chloroplasts were incubated and labelled *in vitro* some of the components of the chloroplast protein pattern were synthesized. From these results it appears that synthesis of most of the protein components of the soluble and membrane fractions is not under immediate control of the nucleus. Since it is unlikely that all these proteins are coded by chloroplast DNA, the genetic messages for at least some of them must be in the form of long-lived messenger RNA from the nucleus. As these researchers point out, however, attempts to isolate messenger RNA from *Acetabularia* have been unsuccessful. Again, the results suggest that at least some of the chloroplast proteins are coded by chloroplast genes.

Despite the lack of evidence to show that specific proteins are coded by chloroplast DNA, and despite evidence that some proteins associated with chloroplasts are under nuclear control, it seems unreasonable that 1.1×10^9 daltons of DNA could exist in a chloroplast and not code for anything. Is it, perhaps, possible that most, if not all, of the chloroplast proteins are in fact coded by chloroplast DNA, but that the expression of chloroplast genes is under the control of the nucleus? Or perhaps some proteins may be coded in the nucleus as well as in the chloroplast DNA. Goffeau (28) suggested this when he stated:

"The fact that the incorporation by isolated chloroplasts is not dependent upon the time of enucleation excludes the participation of a stable mRNA of nuclear origin in our *in vitro* system: if such were the case one would have expected a decay of the activity after long periods of enucleation. This conclusion does not necessarily mean, that *in vivo*, the genesis and replication of chloroplasts does not require the participation of cytoplasmic ribosomes and of nucleus-coded mRNA...However, it seems clear, that after isolation the chloroplasts display only their own independent protein synthesis capacities. In other words, all the proteins which are labelled in our *in vitro* system must be under the control of chloroplast DNA and necessarily synthesized inside the chloroplasts".

If some proteins are coded by genes in the nucleus as well as in the chloroplast, the nuclear genes may be transcribed in whole cells, while the chloroplast genes are repressed. Following enucleation, however, the chloroplast genes may become active. Such a phenomenon could explain the observations of Ceron and Johnson (16) that following enucleation, the overall rate of synthesis of proteins from the soluble fraction showed an initial decrease, but returned to normal levels by 6 weeks post-enucleation. On the other hand, the membrane fraction proteins which show changes during development, but are unaffected by enucleation, may be translated from long lived messenger RNA transcribed in the nucleus. It is also possible, of course, that there is no duplication of chloroplast genes in the nuclear genome. Perhaps some chloroplast proteins are coded by the nuclear DNA, while others are coded by the chloroplast DNA. Zetsche (76) examined the chlorophyll content of nucleate and anucleate cells of *Acetabularia mediterranea* and found that it increases during about 4 weeks after enucleation. From the effects of various inhibitors of RNA and protein synthesis on the increase in chlorophyll content, he concluded that protein synthesis is necessary for chlorophyll

formation or stabilization. Furthermore, Zetsche concluded that the chlorophyll content of the cells is controlled by chloroplast DNA as well as the nuclear genome. It would be of interest to study the DNA:DNA hybridization between the nuclear and chloroplast DNA to determine the extent to which they contain base sequences in common, if at all.

As already mentioned, another alternative is that all of the chloroplast proteins are coded by the chloroplast genome, but the transcription of chloroplast DNA is under nuclear control in intact plants. Control of chloroplast gene expression might be mediated by small molecules, the metabolism of which is under direct nuclear control. Shephard and Bidwell (58) have found that isolated chloroplasts from *Acetabularia mediterranea* carry out normal photosynthesis, as well as the biosynthesis of proteins, pigments, lipids and nucleic acids from inorganic precursors, at the same rates as intact cells. They suggest that the membrane surrounding the chloroplasts is highly selective and that *in vivo* control of chloroplast activity may be mediated by transport mechanisms which govern the rate of entry of some molecules, such as HCO_3^- , and the release of photosynthetic products. Their studies also suggest that indole acetic acid may mediate a control mechanism in intact cells.

Obviously, much more research is required if we are to understand the complex nuclear-cytoplasmic interactions in *Acetabularia*. Although *Acetabularia* chloroplasts exhibit autonomous behavior *in vitro*, the interactions between the nucleus, the chloroplasts and other cellular components must be of some benefit to the organism, otherwise there is no reason to believe that *Acetabularia mediterranea* would have evolved into the complex

cell that it is.

To conclude, *Acetabularia mediterranea* chloroplast DNA has a kinetic complexity of at least $1.1 \pm 0.4 \times 10^9$ daltons. Theoretically, this amount of DNA could code for many, if not all, of the chloroplast proteins. At the present time, however, there is no conclusive evidence to indicate specifically which proteins are coded by the chloroplast genome.

BIBLIOGRAPHY

1. Apel, Klaus, and Hans-Georg Schweiger. 1972. Nuclear Dependency of Chloroplast Proteins in *Acetabularia*. Eur. J. Biochem. 25: 229-238.
2. Askaa, Gerd, C. Christiansen and H. Ernø. 1973. Bovine Mycoplasmas: Genome Size and Base Composition of DNA. J. General Microbiology 75: 283-286.
3. Bak, A. Leth, C. Christiansen and A. Stenderup. 1970. Bacterial Genome Sizes Determined by DNA Renaturation Studies. J. General Microbiology. 64: 377-380.
4. Baltus, E. and J. Brachet. 1963. Presence of deoxyribonucleic acid in the chloroplasts of *Acetabularia mediterranea*. Biochim. Biophys. Acta. 76: 490-492.
5. Bastia, Deepak, Kwen-Sheng Chiang, Hewson Swift, and Peter Siersma. 1971. Heterogeneity, Complexity and Repetition of the Chloroplast DNA of *Chlamydomonas reinhardtii*. Proc. Nat. Acad. Sci. (U.S.A.). 68: 1157-1161.
6. Bayen, Marcel and Andre Rode. 1973. Heterogeneity and Complexity of *Chlorella* Chloroplastic DNA. Eur. J. Biochem. 39: 413-420.
7. Bellamy, A.R. and R.K. Ralph. 1968. Recovery and Purification of Nucleic Acids by Means of Cetyltrimethylammonium Bromide. p. 156-160. In: L. Grossman and K. Moldave [ed.] Methods in Enzymology (XII) Nucleic Acids (B) Academic Press, New York and London.
8. Berger, Sigrid. 1967. RNA-Synthesis in *Acetabularia*. II. RNA-Synthesis in Isolated Chloroplasts. Protoplasma 64: 13-25.
9. Berger, S. and H.G. Schweiger. 1969. Synthesis of chloroplast DNA in *Acetabularia*. Physiol. Chem. Physics. 1: 280-292.
10. Borst, P. 1972. Mitochondrial Nucleic Acids. Annual Review of Biochemistry. 41: 333-376.
11. Britten, Roy J. 1970. Repeated DNA and Transcription p. 187-216. In: E.W. Hanly [ed.] Problems in Biology: RNA in Development. Univ. of Utah Press, Salt Lake City.
12. Britten, R.J. and D.E. Kohne. 1966. Nucleotide sequence repetition in DNA. Carnegie Institution Yearbook 65: 78-106.

13. Britten, R.J. and D.E. Kohne. 1967. Repeated nucleotide sequences. Carnegie Institution Yearbook 66: 73-88.
14. Britten, R.J. and D.E. Kohne. 1968. Repeated Sequences in DNA. Science 161: 529-540.
15. Burton, K. 1956. Study of the Conditions and Mechanism of the Diphenylamine Reaction for the Colorimetric Estimation of Deoxyribonucleic Acid. Biochem. J. 62: 315-323.
16. Ceron, Gabriel, and E. Marshall Johnson. 1971. Control of protein synthesis during the development of *Acetabularia*. J. Embryol. exp. Morph 26: 323-338.
17. Collins, C.H. 1964. Microbiological Methods. Butterworths, London, 330 p.
18. Craig, I.W. and A. Gibor. 1970. Biosynthesis of proteins involved with photosynthetic activity in enucleated *Acetabularia* sp. Biochim. Biophys. Acta. 217: 488-495.
19. Crandall, G. Douglas. 1971. A comparative study of the DNA extracted from chloroplast and nuclear fractions of *Phaseolus vulgaris* L. leaves. Ph.D. Thesis, Indiana University.
20. Crothers, D.M., Neville, R. Kallenbach and B.H. Zimm. 1965. The Melting Transition of Low-Molecular-Weight DNA: Theory and Experiment. J. Mol. Biol. 11: 802-820.
21. DeLey, J. 1969. Compositional Nucleotide Distribution and the Theoretical Prediction of Homology in Bacterial DNA. J. Theoret. Biol. 22: 89-116.
22. Eigner, Joseph, Helga Boedtker and George Michaels. 1961. The thermal degradation of nucleic acids. Biochim. Biophys. Acta. 51: 165-168.
23. Falkow, S. and D.B. Cowie. 1967. Intramolecular heterogeneity of the DNA of temperate bacteriophages. Carnegie Institution Yearbook 66: 88-95.
24. Farber, Florence E. 1969. Studies on RNA metabolism in *Acetabularia mediterranea*. I. The isolation of RNA and labelling studies on RNA of whole plants and plant fragments. Biochim. Biophys. Acta 174: 1-11.

25. Farber, Florence E. 1969. Studies on RNA metabolism in *Acetabularia mediterranea*. II. The localization and stability of RNA species; the effects on RNA metabolism of dark periods and actinomycin D. *Biochim. Biophys. Acta* 174: 12-22.
26. Gibor, A. 1967. DNA Synthesis in Chloroplasts, p.321-328. In T.W. Goodwin [ed.] *Biochemistry of Chloroplasts*, Vol. II. Academic Press. New York and London.
27. Gibor, A. and M. Izawa. 1963. The DNA content of the chloroplasts of *Acetabularia*. *Proc. Nat. Acad. Sci. (U.S.A.)* 50: 1164-1169.
28. Goffeau, A. 1969. Incorporation of amino acids into the soluble and membrane-bound proteins of chloroplasts isolated from enucleated *Acetabularia*. *Biochim. Biophys. Acta*. 174: 340-350.
29. Goffeau, A. and J. Brachet. 1965. Deoxyribonucleic acid-dependent incorporation of amino acids into the proteins of chloroplasts isolated from anucleate *Acetabularia* fragments. *Biochim. Biophys. Acta* 95: 302-313.
30. Green, Beverley R. and Hugh Burton. 1970. *Acetabularia* Chloroplast DNA: Electron Microscopic Visualization. *Science* 168: 981-982.
31. Green, B., H. Burton, V. Heilporn and S. Limbosch. 1970. The cytoplasmic DNAs of *Acetabularia*: their structure and biochemical properties, p. 35-60 In: J. Brachet and S. Bonotto [ed.] *Biology of Acetabularia*, Academic Press, New York and London.
32. Green, B., V. Heilporn, S. Limbosch, M. Boloukhare and J. Brachet. 1967. The cytoplasmic DNA's of *Acetabularia mediterranea*. *Proc. Nat. Acad. Sci. (U.S.A.)* 58: 1351-1358.
33. Haemmerling, J. 1963. Nucleo-cytoplasmic interactions in *Acetabularia* and other cells. *Annual Review of Plant Physiology* 14: 65-92.
34. Heilporn, Viviane and Sylvie Limbosch. 1971. Recherches sur les acides désoxyribonucléiques d'*Acetabularia mediterranea*. (in French, English summary) *Eur. J. Biochem.* 22: 573-579.
35. Kirby, K.S., Elizabeth Fox-Carter and Myrna Guest. 1967. Isolation of Deoxyribonucleic Acid and Ribosomal Ribonucleic Acid from Bacteria. *Biochem. J.* 104: 258-262.
36. Kirk, J.T.O. 1967. Effect of Methylation of Cytosine Residues on the Buoyant Density of DNA in Caesium Chloride Solution. *J. Mol. Biol.* 28: 171-172.

37. Kloppstech, K., and H.G. Schweiger. 1973. Nuclear Genome Codes for Chloroplast Ribosomal Proteins in *Acetabularia* II. Nuclear transplantation Experiments. *Exptl. Cell. Res.* 80: 69-78.
38. Kolodner, Richard, and K.K. Tewari. 1972. Molecular Size and Conformation of Chloroplast Deoxyribonucleic Acid from Pea Leaves. *J. Biol. Chem.* 247: 6355-6364.
39. Laird, Charles D. 1971. Chromatid Structure: Relationship between DNA Content and Nucleotide Sequence Diversity. *Chromosoma* 32: 378-406.
40. Laird, Charles D., Betty L. McConaughy and Brian J. McCarthy. 1969. Rate of Fixation of Nucleotide Substitutions in Evolution. *Nature* 224: 149-154.
41. Mandel, M., Levi Igambi, Janet Bergendahl, M.L. Dodson, Jr. and E. Scheltgen. 1970. Correlation of Melting Temperature and Cesium Chloride Buoyant Density of Bacterial Deoxyribonucleic Acid. *J. Bacteriol.* 101: 333-338.
42. Mandel, M. and J. Marmur. 1968. Use of Ultraviolet Absorbance-Temperature Profile for Determining the Guanine plus Cytosine Content of DNA. p. 195-206. In: L. Grossman and K. Moldave [ed.] *Methods in Enzymology (XII) Nucleic Acids (B)*. Academic Press. New York and London.
43. Mandel, M., C.L. Schildkraut, and J. Marmur. 1968. Use of CsCl Density Gradient Analysis for Determining the Guanine plus Cytosine Content of DNA. p. 184-195. In: L. Grossman and K. Moldave [ed.] *Methods in Enzymology (XII) Nucleic Acids (B)*. Academic Press. New York and London.
44. Marmur, J. 1961. Procedure for the Isolation of Deoxyribonucleic Acid from Micro-organisms. *J. Mol. Biol.* 3: 208-218.
45. Marmur, Julius, and Paul Doty. 1961. Thermal Renaturation of Deoxyribonucleic Acids. *J. Mol. Biol.* 3: 585-594.
46. Miura, Kin-ichiro. 1967. Preparation of Bacterial DNA by the Phenol-pH9-RNases Method. p. 543-545. In: L. Grossman and K. Moldave [ed.] *Methods in Enzymology (XII) Nucleic Acids (A)*. Academic Press. New York and London.
47. Polan, MaryLake, Susan Friedman, Joseph G. Gall, and Walter Gehring. 1973. Isolation and characterization of mitochondrial DNA from *Drosophila melanogaster*. *J. Cell Biol.* 56: 580-589.

48. Puiseux-Dao, S. 1970. *Acetabularia* and Cell Biology. Springer-Verlag. New York Inc. 162 p.
49. Rhaese, Hans-Jurgen and Ernst Freese. 1968. Chemical analysis of DNA alterations. I. Base liberation and backbone breakage of DNA and oligodeoxyadenylic acid induced by hydrogen peroxide and hydroxylamine. *Biochim. Biophys. Acta* 155: 476-490.
50. Reuter, W. and H.G. Schweiger. 1969. Kernkontrollierte Lactat-dehydrogenase in *Acetabularia* [in German, English summary] . *Protoplasma* 68: 357-368.
51. Schildkraut, Carl L., Julius Marmur and Paul Doty. 1962. Determination of the Base Composition of Deoxyribonucleic Acid from its Buoyant Density in CsCl. *J. Mol. Biol.* 4: 430-443.
52. Schweiger, H.G. 1969. Cell Biology of *Acetabularia*. *Current Topics in Microbiology and Immunology* 50: 1-36.
53. Schweiger, H.G., W.L. Dillard, A. Gibor and Sigrid Berger. 1967. RNA-Synthesis in *Acetabularia* I. RNA-Synthesis in Enucleated Cells. *Protoplasma* 64: 1-12.
54. Schweiger, H.G., R.W.P. Master and G. Werz. 1967. Nuclear Control of a Cytoplasmic Enzyme in *Acetabularia*. *Nature* 216: 554-557.
55. Sherphard, D.C. 1965. Chloroplast multiplication and growth in the unicellular alga *Acetabularia mediterranea*. *Experimental Cell Research* 37: 93-110.
56. Shephard, D.C. 1965. An autoradiographic comparison of the effects of enucleation and actinomycin D on the incorporation of nucleic acid and protein precursors by *Acetabularia* chloroplasts. *Biochim. Biophys. Acta.* 108: 635-643.
57. Shephard, D.C. 1970. Axenic Culture of *Acetabularia* in a Synthetic Medium. p. 49-69. In: D.M. Prescott [ed.] *Methods in Cell Physiology*, Vol. 4. Academic Press, New York and London.
58. Shephard, D.C. and R.G.S. Bidwell. 1973. Photosynthesis and Carbon Metabolism in a Chloroplast Preparation from *Acetabularia*. *Protoplasma* 76: 289-307.
59. Shephard, D.C. and Wendy B. Levin. 1972. Biosynthesis in isolated *Acetabularia* chloroplasts. *J. Cell. Biol.* 54: 279-294.
60. Spurr, Arthur R. 1969. A Low-Viscosity Epoxy Resin Embedding Medium for Electron Microscopy. *J. Ultrastructure Research* 26: 31-43.

61. Stanier, R.Y. 1970. Some aspects of the biology of cells and their possible evolutionary significance. p. 1-38. In: H.R. Charles and B.C.J.G. Knight [ed.] Organization and Control in Prokaryotic and Eukaryotic Cells. Cambridge University Press, Cambridge.
62. Stern, Herbert. 1968. Isolation and Purification of Plant Nucleic Acids from Whole Tissues and from Isolated Nuclei. p. 100-112. In: L. Grossman and K. Moldave [ed.] Methods in Enzymology (XII) Nucleic Acids (B). Academic Press, New York and London.
63. Studier, William F. 1965. Sedimentation Studies of the Size and Shape of DNA. *J. Mol. Biol.* 11: 373-390.
64. Stutz, Erhard. 1970. The kinetic complexity of *Euglena gracilis* chloroplasts DNA. *FEBS Letters* 8: 25-28.
65. Stutz, Erhard, and Jobst P. Vandrey. 1971. Ribosomal DNA satellite of *Euglena gracilis* chloroplast DNA. *FEBS Letters* 17: 277-280.
66. Subirana, Juan A., and Paul Doty. 1966. Kinetics of Renaturation of Denatured DNA. I. Spectrophotometric Results. *Biopolymers* 4: 171-187.
67. Tewari, K.K., and S.G. Wildman 1970. Information content in the chloroplast DNA. *Symp. Soc. Exp. Biol.* 24: 147-179.
68. Thrower, K.J. and A.R. Peacocke. 1968. Kinetic and Spectrophotometric Studies on the Renaturation of Deoxyribonucleic Acid. *Biochem. J.* 109: 543-557.
69. Vinograd, Jerome, Robert Bruner, Rebecca Kent and Jean Weigle. 1963. Band-centrifugation of macromolecules and viruses in self-generating density gradients. *Proc. Nat. Acad. Sci. (U.S.A.)* 49: 902-910.
70. Watson, James D. 1970. *Molecular Biology of the Gene*, second edition, W.A. Benjamin, Inc., New York 662 p.
71. Wells, Richard, and Max Birnstiel. 1969. Kinetic Complexity of Chloroplastal Deoxyribonucleic Acid and Mitochondrial Deoxyribonucleic Acid from Higher Plants. *Biochem. J.* 112: 777-786.
72. Wells, Richard, and Ruth Sager. 1971. Denaturation and the Renaturation Kinetics of Chloroplast DNA from *Chlamydomonas reinhardtii*. *J. Mol. Biol.* 58: 611-622.
73. Werz, G., and G. Kellner. 1968. Molecular Characteristics of Chloroplast DNA of *Acetabularia*, Cells. *J. Ultrastructure Research.* 24: 109-115.

74. Wetmur, James G., and Norman Davidson. 1968. Kinetics of Renaturation of DNA. J. Mol. Biol. 31: 349-370.
75. Woodcock, Christopher L.F., and Lawrence Bogorad. 1970. Evidence for variation in the quantity of DNA among plastids of *Acetabularia*. J. Cell Biol. 44: 361-375.
76. Zetsche, K. 1969. Die Wirkung von RNA-und Proteinsyntheseinhibitoren auf den Chlorophyllgehalt kernhaltiger und kernloser Acetabularien. [in German, English summary] Planta (Berl.) 89: 284-298.