THE CYTOPLASMIC DNA'S OF ACETABULARIA MEDITERRANEA*

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The giant alga *Acetabularia mediterranea* has been extensively used for studies of nucleocytoplasmic relationships.[1-3] Anucleate fragments are able to survive and grow for several months, and even to form a species-specific cap. The number of chloroplasts continues to increase after enucleation, although at a slower rate than in nucleate halves.[4] DNA has been shown, by fluorometry, to be present in the chloroplasts of anucleate fragments[5, 6] and to increase in amount with time.[7] Isolated chloroplasts can synthesize RNA[8-12] and must contain many different RNA species, since they are capable of independent protein synthesis.[10]

Acetabularia is particularly interesting from the point of view of chloroplast DNA, because it is the only organism in which the possibility of nuclear contamination of the chloroplast fraction can be definitively eliminated before the start of the experiment. The amount of DNA per chloroplast has been estimated by the above authors,[5] but this DNA was not isolated in its undenatured state, nor was its base composition determined. We have undertaken to isolate the cytoplasmic DNA's of *Acetabularia* and to determine their base composition by buoyant density determination in CsCl. We have found that *Acetabularia* contains two cytoplasmic DNA's, one of which is probably the chloroplast DNA. These DNA's have buoyant densities of 1.704 and 1.714 gm/cc in CsCl, corresponding to 45 per cent and 55 per cent guanosine plus cytidine (G + C) content, respectively. During the course of the work, it became apparent that even "sterile" cultures of this alga contain contaminants, which do not form colonies on nutrient agar, in much greater quantities than expected. Therefore, a considerable part of this work has consisted of electron-microscope controls of cell fractionation.

Materials and Methods.—Cultivation and treatment of algae: Acetabularia mediterranea were cultivated in sterile enriched artificial sea water from cysts removed from caps under sterile conditions.[13] Flasks of 250-ml capacity containing 90 ml sea water and about 50 algae were kept at 21°C and 1800 lux on a 12-hr light cycle. Sea water was changed about every 10 days. The plants used were 2–3 cm in length and free from any visible infection. They were removed to fresh sea water, brushed gently with a watercolor brush to remove possible contaminants attached to the cell wall, and placed for 2–4 days in a solution of 100 mg penicillin, 20 mg neomycin, 20,000 units mycostatin, in 100 ml sterile water.[6] For enucleation, they were put in fresh sea water and sectioned just above the rhizoid with a sharp dissecting knife. After 24–48 hours, they were either removed to fresh sea water or used immediately.

The cysts came from mature caps which had been stored under semianaerobic conditions in the dark at 7°C in normal sea water. Before use, the caps were put in the light for a few days to promote division of the secondary nuclei.

Subcellular fractionation: (1) *Preliminary experiments:* Anucleate fragments (500–800) were cut into small pieces with scissors and ground by hand in a small ground-glass homogenizer in about 10 ml of 0.15 *M* NaCl–0.1 *M* Versene, pH 8.4. Sedimentation of chloroplasts and membranes was effected by 10 min in a Sorvall centrifuge (SS-34 type) at 12,000 *g*.

(2) *Differential centrifugation:* From 1000 to 5000 anucleate fragments were ground in 10–15 ml of 0.4 *M* mannitol, 0.02 *M* Tris, 0.01 *M* NaCl, 0.05 *M* Versene, pH 8.4. The volume of the

solution was kept as low as possible. A high concentration of Versene was used to inhibit DNases, since the intracellular concentration of Mg^{++} ions is extremely high.[14] The homogenate was filtered through a single layer of bolting silk to remove cell walls and other debris. The bulk of the chloroplasts was sedimented after 7 min at 2400 *g* in a Hettich desktop centrifuge (fraction I). The pale green supernatant was spun 15 min at 10,400 *g* in the Sorvall HB-4 swinging-bucket rotor, giving a green pellet (fraction II). The supernatant from this step was spun 30 min at 56,590 *g* in the Spinco 40 rotor, giving another green pellet (fraction III).

(3) *Sucrose gradient:* After filtration through bolting silk, the homogenate of 1500 algae (5–6 ml) was layered directly on a discontinuous gradient of 5 ml each of 2 *M*, 1.5 *M*, 1.25 *M*, and 1 *M* sucrose in 0.01 *M* Tris, pH 7.5. After 30 min at 22,500 rpm in the Spinco SW-25 rotor (51,505 *g*), the bulk of the chloroplasts remained at the interface between the 0.4 *M* mannitol and the 1.0 *M* sucrose. This layer was removed and washed twice with homogenization medium.

Extraction of DNA from fractions: DNA was extracted from subcellular fractions by Marmur's method[15] with dialysis overnight against saline-citrate instead of precipitation. Yields were variable, but were of the order of 10 μg DNA/1000 algae as measured by optical density at 260 mμ.

DNA from cysts: From 40 to 100 healthy mature caps which had not started to liberate their cysts were used for each experiment. The caps were cut up in sea water, passed through a nylon tea strainer, and collected on bolting silk. A better method consisted of cutting the caps in two, squeezing, and scraping off the cysts with a sterile spatula. Especially when combined with extensive washing by decantation, this method eliminates most of the microbial contamination inevitably found in the caps.

The washed cysts were homogenized in a motor-driven Teflon glass homogenizer with 2 ml of 5% sodium dodecyl sulfate (SDS) in 0.15 *M* NaCl–0.1 *M* Versene, pH 8.5, heated to 65°C, for 5 min, and the isolation of DNA continued as above.

CsCl density gradients: About 0.8 ml of the solution containing 1–5 μg/ml DNA was made up to a density of 1.70 gm/cc with the addition of solid CsCl. DNA of *Micrococcus lysodeikticus* (ρ = 1.731 gm/cc) or LP7 phage (ρ = 1.741 gm/cc) was added as density reference. The solution was centrifuged at 44,770 rpm for 20 hr in a standard 12 mm Kel-F cell in the AN-D rotor of a Spinco model E. Photos were taken at 265 and 420 mμ and scanned with a Joyce-Loebl microdensitometer. Buoyant densities were calculated according to Schildkraut *et al.*[16] without correction for pressure effects. Base compositions were calculated from the formula ρ = 1.660 + 0.098 (G + C) gm/cc^{-3}.[16]

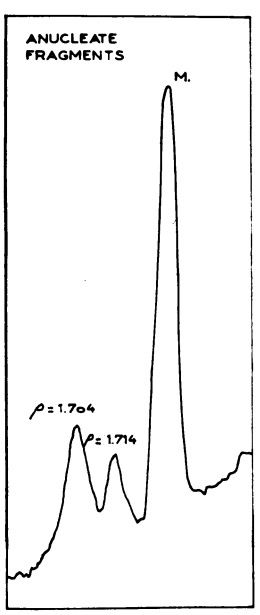

FIG. 1.—CsCl density gradient centrifugation of DNA from anucleate fragments of *Acetabularia mediterranea*. *M* = *Micrococcus lysodeikticus* DNA reference (ρ = 1.731 gm/cc).

Electron microscopy: The different pellets, collected in the Sorvall centrifuge, were fixed in a 6% solution of glutaric aldehyde in Millonig phosphate buffer, pH 7.4, containing 0.25 *M* sucrose. Fixation was followed by superfixation in 1% osmium tetroxide in Millonig buffer, pH 7.4. The pellets were dehydrated and embedded in araldite. Sections were made in an LKB microtome, type II. They were stained with both uranyl acetate and lead citrate. The grids were examined on an AEI type EM6B electron microscope.

Results.—(1) *Anucleate fragments:* (a) *Preliminary experiments:* In these experiments, the homogenate was simply spun down at 12,000 × *g*, giving a pellet which contained chloroplasts and membranes and would be expected to contain mitochondria as well. The pellet yielded two DNA's of density 1.704 and 1.714 gm/cc, corresponding to 45 and 55 GC content (see Fig. 1). When 1000 whole cells were ground in the mannitol homogenization medium, filtered through bolting silk, and sedimented at 12,000 × *g* for 15 minutes, the pellet yielded the same two DNA's. In this case, one would not expect

to see the nuclear DNA because there are only 1000 nuclei. In both types of experiment, the relative amount of material in the two peaks varied from one time to the next, but they were approximately equal. Both peaks were sensitive to DNase (50 μg/ml for 3 hr at 37°C). When denatured by boiling for ten minutes, they gave a single broad peak of density 1.724 gm/cc.

(b) *Differential centrifugation:* Fraction I, when examined with the electron microscope, was found to consist of large chloroplasts, many of which had mitochondria absorbed to them or attached by cytoplasm (Fig. 2). The DNA of this fraction is strongly enriched for the lighter peak (1.704 gm/cc), but still contains some of the 1.714 gm/cc peak (Fig. 3).

Heat denaturation results in two separate peaks which have increased in density

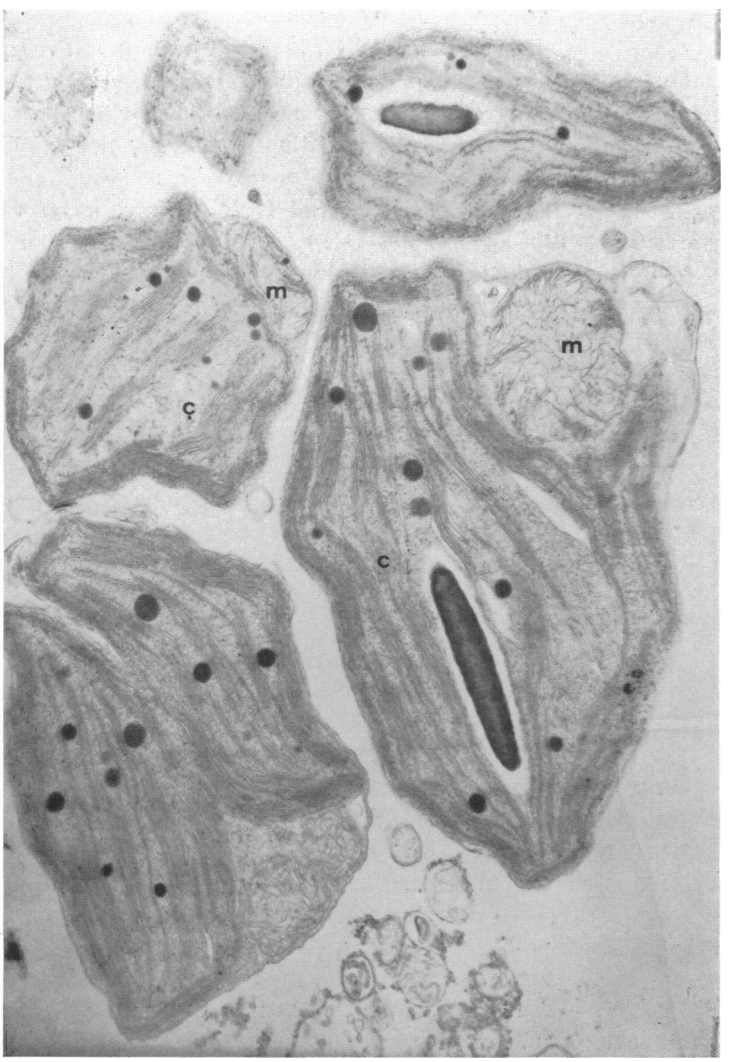

FIG. 2.—Fraction I. Chloroplasts (*c*) with associated mitochondria (*m*). ×18,750.

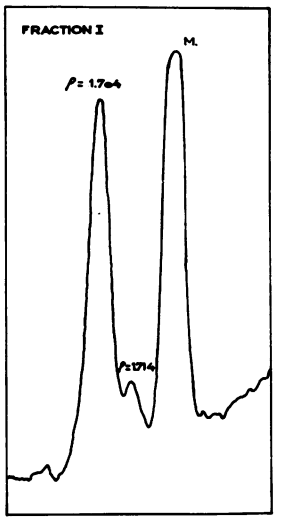

FIG. 3.—CsCl density gradient centrifugation of fraction I DNA. *M* = *M. lysodeikticus* DNA (ρ = 1.731 gm/cc).

by 0.018 and 0.016 gm/cc, respectively. Since most of the chloroplasts are sedimented at this speed (2400 × *g*), which is probably not high enough to bring down the bulk of the mitochondria, it is most likely that the 1.704 gm/cc DNA is the chloroplast DNA.

Fraction II contained several peaks, all of which were sensitive to DNase. The major peak had a density of 1.698 gm/cc. Electron micrographs showed the presence of a large number of contaminating microorganisms of typically gram-negative appearance, as well as mitochondria and some small chloroplasts (Fig. 4). In an experiment where 5000 algae were used, half of which had been enucleated for more than one week, peaks of density 1.698, 1.713, and 1.722 gm/cc were found (Fig. 5).

Since the contaminant is predominant when 1.698 gm/cc is the major DNA, it can be concluded that it is the DNA of the contaminant. The peak at 1.714 gm/cc was present in all preparations, as would be expected if it were mitochondrial DNA. There is no visible peak of chloroplast DNA, probably because there is only a small number of chloroplasts in this fraction. The 1.722 gm/cc peak was found in both fraction I and fraction II of this particular experiment, and not at all in two other experiments where smaller numbers of algae were used.

Fraction III consisted of empty vesicles and islands of cytoplasm, some of which contained ribosomes. It did not contain detectable amounts of DNA, nor did washed membranes from the filtration step. This shows that any attached microorganisms were removed either by the brushing or by the homogenization.

(c) *Sucrose gradient:* The washed chloroplast pellet from the sucrose gradient yielded two peaks of DNA, one at 1.703 gm/cc and the other at 1.722 gm/cc (see Fig. 6).

Electron-microscope examination of this fraction showed only large chloroplasts, somewhat swollen, and many well-preserved mitochondria. No contaminating microorganisms were seen, a finding which is consistent with the absence of the 1.698 gm/cc peak.

(2) *Cysts:* In four experiments, where small numbers of caps were used or where the cysts were removed under sterile conditions and washed carefully, a rather broad peak was found (Fig. 7). It had an average buoyant density of 1.702 gm/cc.

(3) *Culture contaminants:* When DNA is extracted from rhizoids, at least five peaks are obtained. This is not surprising, considering the impossibility of removing contaminating microorganisms from the irregular surface of the rhizoid.

If the medium in which the algae were brushed or enucleated is kept for some days, a considerable pellet of microorganisms can be obtained by centrifugation. This pellet also yields a number of DNA's, the densities of which are very close to those obtained from the rhizoids: ρ = 1.696, 1.704, 1.713, 1.723, 1.731 gm/cc.

Discussion.—The DNA density 1.704 (± 0.001) gm/cc is almost certainly the

chloroplast DNA, since it becomes the major peak when the homogenate is enriched for chloroplasts by centrifugation. This corresponds to a base composition of 45 per cent G + C. It should be noted that the base composition (determined by chromatography) of the RNA isolated from whole chloroplasts of *Acetabularia*[17] is 42 per cent G + C.

It is likely that the 1.714 (±0.001) gm/cc DNA is the *mitochondrial* DNA, since it is found in fractions I and II, both of which are seen to contain mitochondria in the electron microscope. Moreover, the mitochondria are the only particles, in addition to chloroplasts, found in fraction I. Only very occasionally were bacterial contaminants found in any chloroplast fraction. This peak was not seen in the chloroplast fraction of the sucrose gradient experiment (Fig. 6), although it does contain some mitochondria; but it is possible that this mitochondrial DNA was present in such small quantity that it did not show when only a small amount of DNA was put on the gradient.

When the two cytoplasmic DNA's are present in about equal amounts, they seem

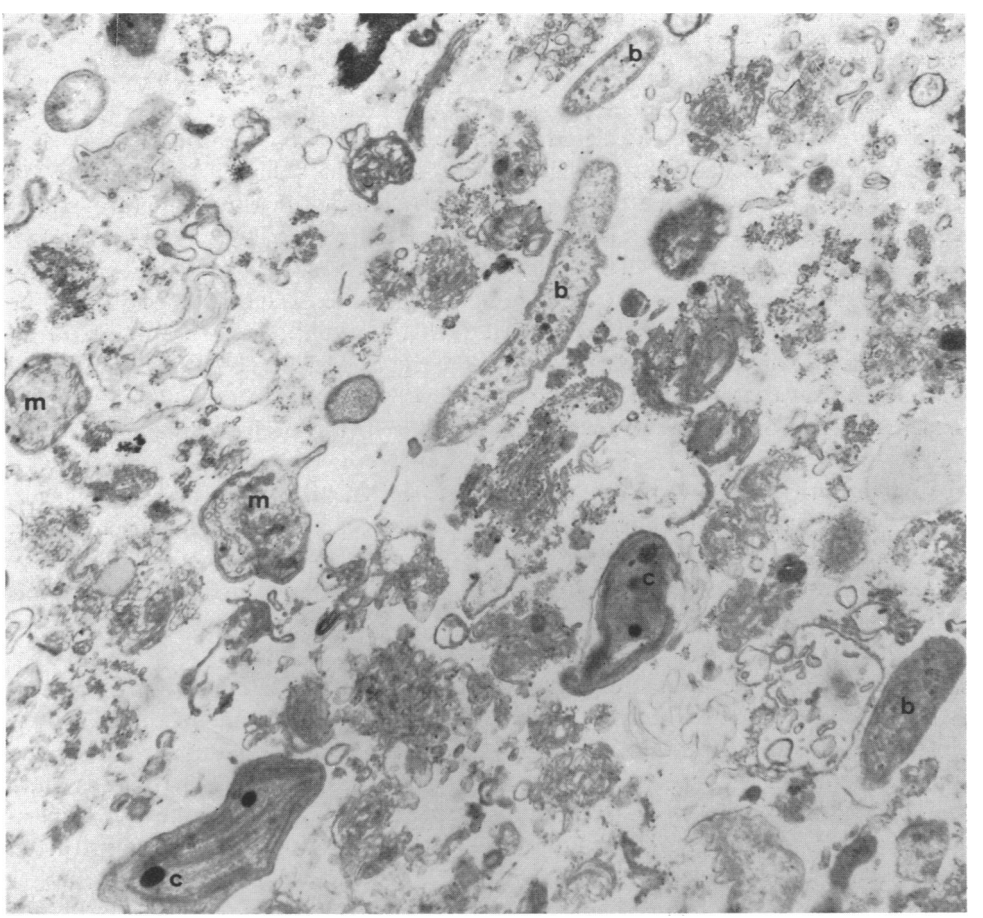

FIG. 4.—Fraction II. Small chloroplasts (*c*), mitochondria (*m*), and contaminating microorganisms (*b*). ×18,750.

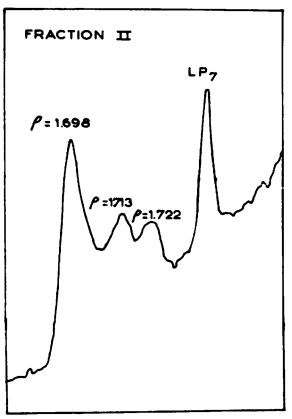

FIG. 5.—CsCl density gradient centrifugation of fraction II DNA with LP7 phage DNA as density reference (ρ = 1.741 gm/cc).

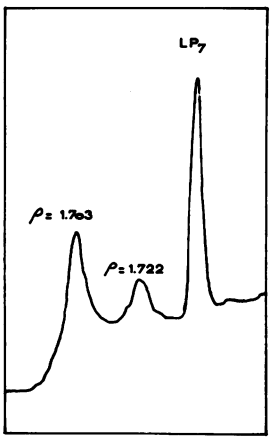

FIG. 6.—Sucrose gradient experiment. CsCl density gradient centrifugation of DNA from chloroplast pellet. LP7 DNA (ρ = 1.741 gm/cc).

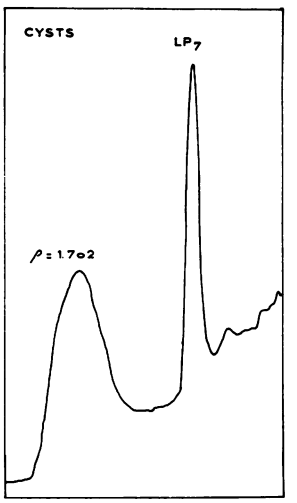

FIG. 7.—CsCl density gradient centrifugation of cyst DNA. LP7 DNA (ρ = 1.741 gm/cc).

to interfere with each other's denaturation, since they give one broad band at a density nearer that of denatured chloroplast DNA than that of denatured 1.714 gm/cc DNA. This unusual behavior has been observed in three separate experiments. When there is only a small amount of 1.714 gm/cc DNA present, the two DNA's denature normally. There is no obvious explanation for this phenomenon.

The origin of the 1.722 gm/cc peak found in the later experiments remains to be explained. If it were the mitochondrial DNA, it should have been seen in all fractions containing mitochondria. It has the right density to be denatured chloroplast DNA, but the preparation were not subjected to conditions commonly known to denature DNA. A recent experiment similar to the preliminary ones yielded DNA peaks at 1.698 and 1.723 gm/cc in addition to the two already found (Fig. 1); this suggests that this peak is due to a new contaminant in the cultures. However, if so, at least half as many microorganisms as chloroplasts should have been seen in the micrographs of the sucrose gradient experiments (Fig. 6), whereas none were found. There remains the possibility that this DNA might be due to a virus, but no structures resembling virus particles were seen in any electron micrograph examined.

As is very evident from the above, the question of bacterial contamination of "sterile" cultures is a serious one. The 1.698 gm/cc DNA can definitely be attributed to the microorganism seen in Figure 4. Since many bacteria have the same appearance under the electron microscope, it is always possible that there is more than one species present. The contaminant peak was not seen in the early experiments, probably because smaller numbers of algae were used which were extracted within 48 hours of enucleation, so that infection did not have time to develop.

The homogenate from one of these experiments was plated on nutrient agar at different dilutions, but gave almost no colonies on incubation either at room temperature or at 37°. However, microorganisms with special growth requirements, slow-

growing autotrophs, or symbionts might not form colonies on tryptone agar; so these negative results are not necessarily a proof of sterility.

Gibor[18] has recently published the results of his experiments with DNA from anucleate *Acetabularia* which had been labeled for nine days with $C^{14}O_2$. He identifies the chloroplast DNA with the most highly labeled peak, which has a density of 1.695 gm/cc. He does not report having looked at the total DNA of anucleate fragments in the analytical ultracentrifuge, nor examining his chloroplasts under the electron microscope. From our own experience, and in the absence of further information, it seems that the 1.695 gm/cc DNA is more likely to be the DNA of a contaminant which was able to grow enough in nine days to become well labeled, but not enough to make the infection visible under the light microscope. The only valid method of ascertaining if a subcellular fraction is sterile is to examine it with the electron microscope.

It cannot as yet be definitely concluded that the nuclear DNA of *Acetabularia* has a density of 1.702 gm/cc. Since there are many more chloroplasts than nuclei in a cyst,[19] it is possible that the single broad peak found in the "clean" experiments is really a double peak of chloroplast and contaminant DNA. However, using quite a different method (electrophoresis on a silk thread), Baltus *et al.*[17] found that the DNA of *Acetabularia mediterranea* gametes has a base composition of 42 per cent GC which corresponds exactly to a buoyant density of 1.702 gm/cc.

Summary.—Anucleate fragments of *Acetabularia mediterranea* contain two DNA's. The chloroplast DNA has a buoyant density in CsCl of 1.704 ± 0.001 gm/cc, which corresponds to a 45 per cent GC content. The other cytoplasmic DNA, which may be mitochondrial, has a buoyant density of 1.714 ± 0.001 gm/cc, corresponding to a 55 per cent GC content. A single broad peak of density 1.702 gm/cc was found in the cysts; this may or may not represent the nuclear DNA. The interpretation of the results is complicated by the frequent presence of bacterial contaminants.

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