

EVIDENCE FOR VARIATION IN THE QUANTITY OF DNA AMONG PLASTIDS OF *ACETABULARIA*

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ABSTRACT

The DNA content of individual plastids of the giant unicellular algae *Acetabularia mediterranea*, and *Polyphysa cliftoni* was studied. Four methods were used for localizing DNA: acridine orange staining, radioautography following actinomycin D-³H treatment, electron microscopy of thin tissue sections, and electron microscopy of osmotically disrupted plastids. With each method, DNA was readily detected in 20–35% of plastids, but no DNA was observed in the remaining 65–80%. The results further showed that in those plastids with detectable DNA the amount of DNA present was variable. The sensitivity and reliability of the localization methods are discussed, and the possible implications of these findings are considered.

INTRODUCTION

Although DNA has been detected in intact plastids by staining, radioautography, and electron microscopy (17, 18), most of the information on physical and chemical properties of plastid DNA has been gained not from such *in situ* studies, but from the examination of DNA isolated from large numbers of plastids. Many valuable data concerning the nature and quantity of plastid DNA have been obtained with the latter approach, but the results are necessarily averages of the total plastid DNA population; variation in DNA content among plastids cannot be observed. The present investigation was designed to determine the DNA content of individual plastids and to study the morphology of plastid DNA, though this study is chiefly concerned with the former objective.

When plastids are examined on an individual basis for the presence of DNA, it is vitally important to ensure that they are uncontaminated with other DNA species; the difficulty of isolating plastids free of nuclear DNA has been amply

demonstrated for most plants (7, 17). In the case of the giant alga *Acetabularia*, however, the single nucleus remains at the basal end of the cell during vegetative growth (13) and hence, the preparation of uncontaminated plastids is a simple matter. During an investigation into the DNA content of individual *Acetabularia* plastids, we observed marked variations in DNA quantity from plastid to plastid. The validity of this observation has been tested by using a number of methods of detecting DNA *in situ*. Evidence will be presented which demonstrates the following: (a) there is a wide variation in the quantity of DNA/plastid among plastids in which DNA is clearly and easily detected; and (b) there is a class of plastids, comprising 65–80% of the population, in which DNA could not be detected by any of the methods used.

MATERIALS AND METHODS

Stocks of *Acetabularia mediterranea* were kindly provided by Dr. A. Gibor (who also supplied *Polyphysa cliftoni*),

Mme. S. Puiseux-Dao, and Dr. J. Brachet. They were maintained in Erdschriber medium (12) or in the defined medium of Shephard (31) at 70°F and were illuminated with 300–400 ft-c of mixed fluorescent and incandescent light for 12 hr/day.

For fluorescence microscopy, plants 1–4 cm long and without caps were enucleated, and the cell contents were withdrawn from the stalk with a fine capillary. The contents were expelled immediately into a drop of formalin-acetic acid fixative (16), mixed thoroughly, and transferred to gelatin-coated glass microscope slides. After drying, the slides were washed in 70% ethanol, transferred to 100% ethanol, and air dried. Alternatively, the cell contents were fixed, and the plastids were prepared by the method of Shephard (30), in which formalin fixation is followed by differential centrifugation, before the plastids are transferred to slides. For preparations of cap plastids, young caps were chopped briefly in a drop of fixative, and the cell exudate was treated as above. After the slides were placed for 5 min in 0.2 M acetate buffer pH 3.0–3.2, they were stained in 0.05% acridine orange (Allied Chemical C.I. No. 40065, Allied Chemical Corp. National Aniline Div., Boston, Mass.) in the same buffer for 5–15 min, rinsed thoroughly in buffer, and examined immediately (3). The microscope (Leitz, Ortholux) was equipped with a xenon lamp, blue BG 12 and BG 3 excitation filters, an oil immersion dark field condenser, and an oil immersion 90× objective lens fitted with an iris diaphragm. An orange filter (Absorption minimum 530 nm) prevented blue light from reaching the eyepiece. Photographs were recorded on High Speed Ektachrome (Kodak) or Plus X Pan (Kodak) film.

Control slides were incubated with DNase (Worthington Biochemical Corp., Freehold, N. J., DPFF, ribonuclease-free) at a concentration of 0.2 mg/ml in 0.005 M MgSO₄ brought to pH 6.5, or ribonuclease (Worthington Biochemical Corp.) at 0.2 mg/ml in 0.01 M phosphate buffer pH 7.0, or in the incubation media alone. All digestions were carried out at 37°C for 3 hr.

Labeling with tritiated actinomycin D was carried out on formalin-acetic acid-fixed plastids following the method of Camargo and Plaut (6). Actinomycin D-³H (Schwarz Bio Research Inc., Orangeburg, N. Y.) was diluted with water to 25 μCi/ml, and 4 drops (0.1 ml) were placed on each slide containing plastids. Cover glasses were loosely applied, and the slides were placed in a water-saturated atmosphere for 60 min. After this time, the cover glasses were removed, and the slides were washed three times in water, three times in 50% ethanol, three times in 70% ethanol, transferred to 100% ethanol, and air dried. Control slides were treated with nucleases before or after treatment.

¹L₄ nuclear emulsion (Ilford Ltd., Essex, England) was dissolved in water in 1:3 w/v ratio at 45°C, and

the labeled slides were dipped. After 20–25 days, the slides were developed in D 19 developer (Kodak) for 4 min at 18°C, transferred to 2% acetic acid for 30 sec, fixed in hypo (Kodak Fixer) for 5 min, and were washed thoroughly before examination.

The method used for the spreading of DNA released by plastids during osmotic disruption was based on the technique of Kleinschmidt et al. (19) as modified by Nass for the preparation of mitochondrial DNA (22). Cell contents were removed from enucleated *Acetabularia* as described above and were mixed with ammonium acetate and cytochrome *c* to final concentrations of 0.5 M and 400 μg/ml, respectively. The drop of plastid suspension was transferred to a clean stainless steel needle, which was then touched to a clean water surface (36). The resulting circular protein film, 1–3 cm in diameter, was picked up on grids coated with carbon and Formvar and dried from ethanol. In some cases DNase or self-digested Pronase (Calbiochem) was added at final concentrations of 25 and 100 μg/ml, respectively, before spreading. Alternatively, a drop of DNase solution containing 100 μg of enzyme/ml was added to the specimen on the grid for 1–5 min before drying.

Platinum shadowing was performed in an evaporator (Kinney Vacuum, Model KSE-2, New Air Brake Co., Boston, Mass.) at a vacuum of 10⁻⁶ torr. 1–2 cm platinum wire was evaporated from each of two heavy tungsten filaments at right angles to each other and at an angle of 5–7° to the specimen. The grids were examined with a Hitachi HU 11 C electron microscope, and micrographs were taken at 5,000–10,000 magnification. A diffraction grating replica was used for calibration. In some cases, the length of DNA strands was estimated on projected images of negatives by using a map measurer.

For the fixation of whole cells glutaraldehyde (final concentration 5%) purified with charcoal (2) was used either in 0.1 M phosphate buffer, pH 7.2 containing 25% seawater, or in 0.05 M Veronal-acetate buffer, pH 6.0 containing 0.6 M NaCl and 0.005 M CaCl₂ (23).

The slight precipitate which often formed in the phosphate-buffered fixative was centrifuged out before use. After fixation for 2 hr at 0°C, the cells were washed thoroughly with the appropriate buffer before postfixation in 1% OsO₄ in water for 30 min. Following a further wash, cells that had been fixed in Veronal buffer were treated for 45 min with 0.5% uranyl acetate in buffer (23) before acetone dehydration, while the cells fixed in phosphate buffer were dehydrated to 75% acetone before staining in 1% uranyl acetate in 75% acetone. From pure acetone the specimens were transferred to propylene oxide and then by stages to Durcupan ACM (Fluka AG, Basel, Switzerland). Polymerization was carried out at 60°C for 48 hr and, after trimming, the blocks were sectioned on a Porter-Blum MT-2 ultramicrotome

(Ivan Sorvall Inc., Norwalk, Conn.). Lead citrate (26) was used to stain sections before examination. For serial sections, gold-silver ribbons were picked up on single hole slit grids coated with carbon-Formvar film. Undamaged grids containing 20–30 sections in two or more ribbons were selected for electron microscopy.

Due to the folding of some sections, the assembled serial photomicrographs of individual plastids were approximately 80% complete. After assembly, each series was checked by examination of the original negative for the presence of 25 A fibrils.

RESULTS

Fluorescence Microscopy

A sensitive histochemical test for nucleic acids is their fluorescence after staining with acridine orange. In the pH range 3–5 and at suitable dye concentrations, the complex formed between acridine orange and double-stranded DNA emits a yellow-green fluorescence, whereas the corresponding RNA complex fluoresces red (3). This technique was used by Ris and Plaut (27) in the successful detection of plastid DNA in *Chlamydomonas*.

After formalin-acetic acid fixation and acridine orange treatment, *Acetabularia* plastids showed an overall faint greenish-yellow background fluorescence, which could not be removed by DNase or RNase digestion. A similar faint fluorescence was observed in fixed but unstained plastids. In addition, some plastids contained one or occasionally two yellow-green, brightly fluorescing areas of varying size and intensity (Fig. 1). After formalin fixation, which did not remove plastid pigments, the bright red chlorophyll fluorescence was retained, and after acridine orange staining, the yellow-green DNA regions were seen in sharp contrast (Figs. 3–5). In such preparations, DNA fluorescence was emitted only from within bodies showing red chlorophyll fluorescence, although other organelles were present. The latter could be seen only in phase contrast and, from their size, were tentatively identified as mitochondria. Treatment with DNase abolished the yellow-green fluorescing regions (Fig. 2) whereas treatment with enzyme-free digestion medium or RNase did not.

Counts of the number of plastids exhibiting yellow-green fluorescing spots were made on preparations from various regions of *A. mediterranea* and *P. cliftoni* plants of a variety of ages. The results are shown in Table I. Despite vari-

ations in size, maturity, and species, the proportion of plastids showing fluorescent regions was relatively constant, falling between 20 and 35% in most cases.

Transfer of plants from enriched seawater to the defined medium did not affect the percentage of plastids exhibiting DNA fluorescence. An abnormal metabolic state caused by malnutrition, therefore, seems an unlikely explanation of our observations.

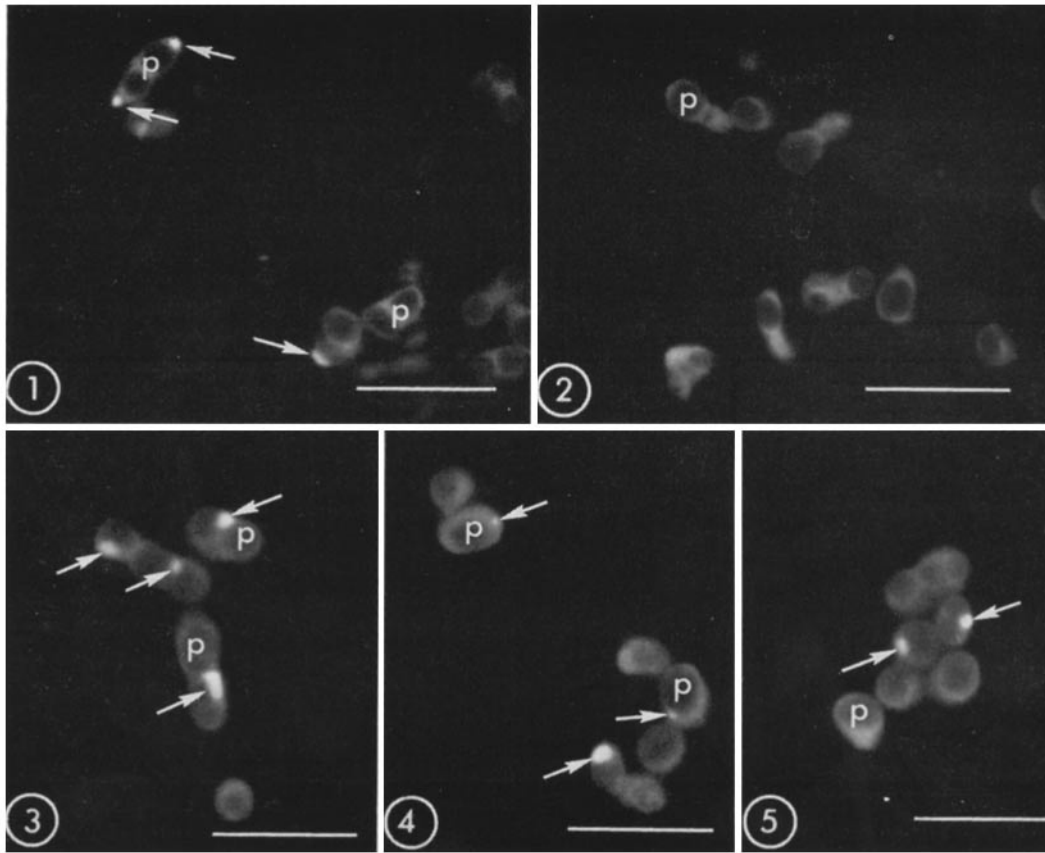
Although acridine orange fluorescence has been shown to be a highly sensitive method for DNA detection (1), this sensitivity is only realized when the DNA is in a sufficiently compact form. In studies on the basal body of *Tetrahymena pyriformis*, which were estimated to contain 2×10^{-16} g DNA, Randall and Disbrey (24) showed that acridine orange fluorescence was readily detected in most cases. At one stage in the division cycle, however, fluorescence disappeared, and it was suggested that this was caused either by dispersion of the DNA at replication, or by masking of the DNA by basic proteins. The latter effect was considered unlikely, since DNA fluorescence is unimpaired in chromatin. The possibility that the failure to detect DNA by acridine orange fluorescence in 65–80% of *Acetabularia* plastids was due to similar phenomena, has been examined by the use of DNA detection methods that are unaffected by dispersion or by masking.

Radioautography Following In Vitro Actinomycin D-³H Labeling

The binding of actinomycin D to DNA in vitro is well established (25), and the radioactively labeled form of the antibiotic has been used in conjunction with radioautography to localize DNA in the nucleoli of *Drosophila* and the kinetoplast of *Leishmania* (6). The technique should be equally sensitive in detecting condensed or dispersed DNA.

After treatment with actinomycin D-³H, formalin-acetic acid-fixed plastids of both *A. mediterranea* and *P. cliftoni* were not uniformly labeled. Only one or two silver grains were localized over the majority of plastids, but the remaining plastids were associated with several grains, often localized in one or occasionally two regions (Figs. 6–10).

With prior DNase treatment, these groups of localized grains were not seen (Fig. 11). In order to allow for the effect of background and non-specific labeling, counts were made on both con-



FIGURES 1 and 2 Fluorescence of *Acetabularia mediterranea* plastids after acridine orange staining. Fig. 1 untreated; Fig. 2 DNase treatment. Arrows indicate brightly-fluorescing DNA-containing areas. *p*, plastid. Bar = 10 μ . \times 1,600.

FIGURES 3-5 Fluorescence of *Polyphysa cliftoni* plastids after acridine orange staining. Arrows indicate brightly fluorescing DNA-containing areas. Fluorescing regions show a wide variation in size and intensity. *p*, plastid. Bar = 10 μ . \times 1,600.

trol and DNase-treated plastids, and these results are presented in histogram form (Fig. 12). To estimate the proportion of plastids with significant label, the two histograms were superimposed, and the area of the control histogram lying outside the DNase histogram was calculated (shaded area in Fig. 12).¹

By this method, the approximate proportion of plastids having significant label was calculated for a number of experiments; the results are shown in Table II. The number of plastids showing significant label is again similar in the two species and falls between 25 and 35%.

From the acridine orange data, two possible

¹ See Note 1 Added in Proof.

labeling patterns after actinomycin D-³H treatment could be predicted for *Acetabularia* plastids. If the lack of fluorescence in 65-80% of plastids were a result of dispersion of the DNA within the stroma, then all plastids should be labeled, but in 20-35% of plastids the grains should be localized in one region. On the other hand, if the fluorescence data reflect a real difference in DNA content, then 20-35% of plastids should be more highly labeled than the rest, and the label should be localized within each plastid. From Figs. 6-10 and Table II it is seen that the latter prediction is fulfilled in the case of both *A. mediterranea* and *P. cliftoni*. Although 65-75% of plastids had an

insignificant number of silver grains, it is, of course, possible that they possessed DNA in too small a quantity to be detected.

In order to correlate the light microscopic evidence for DNA-containing areas in *Acetabularia* plastids with direct ultrastructural observation, and to show that these areas were located *inside* the plastids, serial sections of glutaraldehyde-fixed cells were prepared.

Electron Microscopy of Sections

After glutaraldehyde fixation, aggregates of fibrils were seen in plastids of *A. mediterranea*,

of serial sections; it had already been shown (Table I) that in plants of this size acridine orange staining of the plastids showed a pattern similar to that of more mature cells.

In serial sections, fibrillar regions occurred in some plastids (Fig. 17) but could not be detected at all in others (Fig. 18). Since the characteristic fibrillar regions always occurred in three to six adjacent sections, it was unlikely that their absence in other plastids was due to the loss of about 20% (two to four) of nonconsecutive sections in each series. Careful examination of the plastids in which no fibrillar aggregates occurred failed

TABLE I
Acridine Orange Fluorescence of Plastids

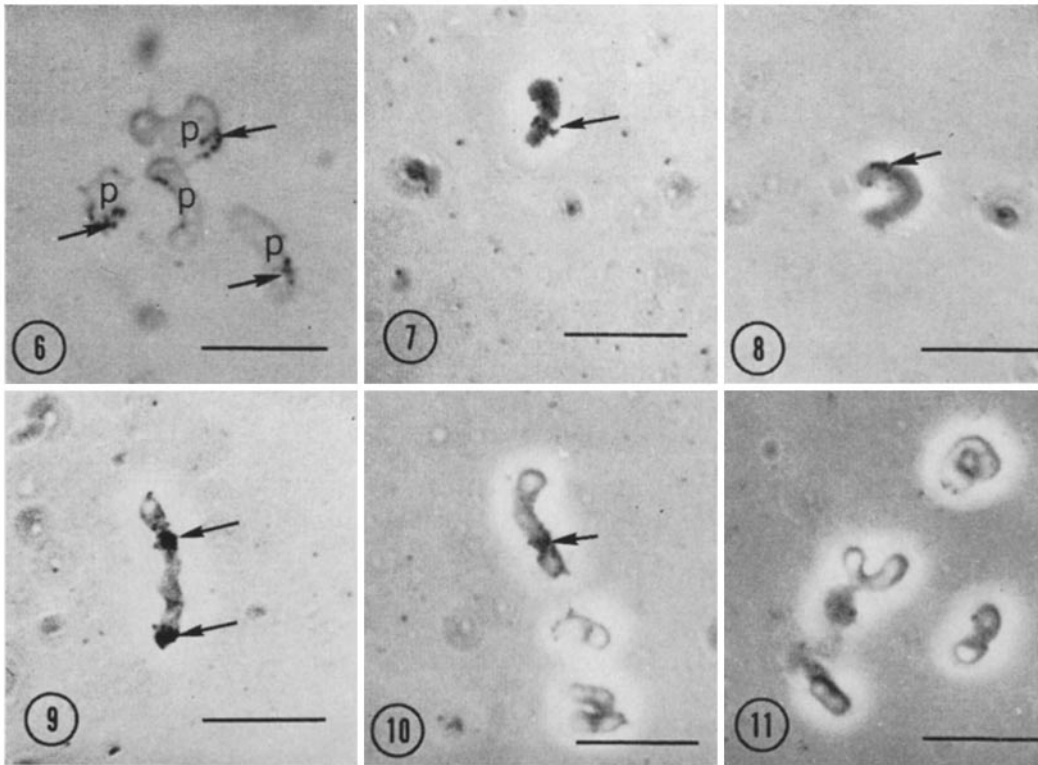
Species	Source of plastids	No. plastids showing DNA fluorescence	Total No. plastids	% of plastids showing DNA fluorescence
<i>Acetabularia mediterranea</i>	Stalk, 1-3 cm long	39	201	19
		90	364	25
		40	283	14
		26	121	22
		97	278	35
	Stalk, 0.5 cm long Rhizoid Young cap, 2-5 mm diameter	179	600	30
		88	296	30
		21	78	27
		86	379	19
				Mean 25
<i>Polyphysa cliftoni</i>	Stalk, 2-4 cm long	37	139	27
		118	554	21

which were similar to those identified as DNA by Puiseux-Dao et al. (23) and by Werz (32) (Figs. 13-16). The smallest fibril diameter was about 25 A. Fibrillar regions occurred in less dense portions of stroma and were not closely associated with plastid membranes.

Although the Ca^{++} -enriched Veronal buffer gave better preservation of membranes and ribosomes, the contrast of the fibrillar region was less than that obtained with phosphate-buffered glutaraldehyde. The latter fixation was, therefore, used in the preparation of serial sections. The quality of preservation of the cells deteriorated as the size of the plant increased, possibly due to the relative increase in the volume of the highly acidic vacuole. Similarly, the sectioning properties deteriorated as plant size increased, and this was attributed to the increased wall thickness and calcification. Consequently, small plants about 5 mm long were chosen for the preparation

to reveal the presence of 25 A diameter fibrils in any portion of the stroma.

Table III shows that DNA-containing regions were present in about 20% of serially sectioned plastids, which is in good agreement with the values obtained with light microscopic techniques. The direct correlation between the presence of localized regions of DNA in plastids as seen in the fluorescence and radioautographic studies and the distinctive structures seen with the electron microscope excludes the possibilities that the former observations were due to surface contaminants of plastids, or to the preferential activity of endogenous DNases during plastid isolation. It remains a possibility that in the 80% of plastids in which DNA areas were not observed, DNA was present but in a form other than a condensed fibrillar mass. Although the stroma of these plastids was carefully examined for individual, 25 A fibrils without success, it is clear



FIGURES 6-11 Radioautographs of plastids (*p*) of *Polyphysa cliftoni* after incorporation of actinomycin D-³H. Figs. 6-10 show untreated plastids with groups of silver grains (arrows). In Fig. 11 the preparation was treated with DNase prior to application of the emulsion. Bar = 10 μ . \times 1,700.

TABLE II
Radioautographic Data From Actinomycin D-³H-
labeled Plastids

Species	No. plastids showing significant label	Total No. plastids	% of plastids showing significant label
<i>Acetabularia mediterranea</i>	37	127	33
	25	84	30
<i>Polyphysa cliftoni</i>	25	88	28
	22	84	26

that strands closely associated with membranes would escape detection.

No correlation was observed between the presence of a DNA-containing region in a plastid and its size, shape, number of inulin grains, or abundance of ribosomes.

It is not possible with the above techniques to measure the amount of DNA present, or to de-

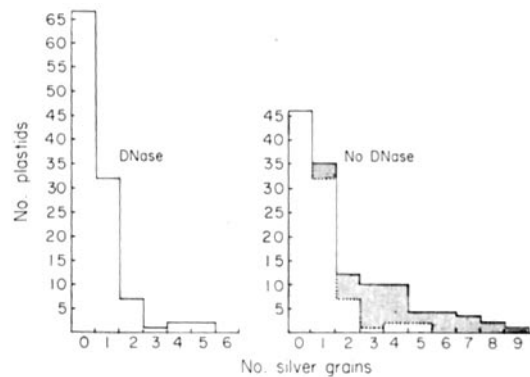
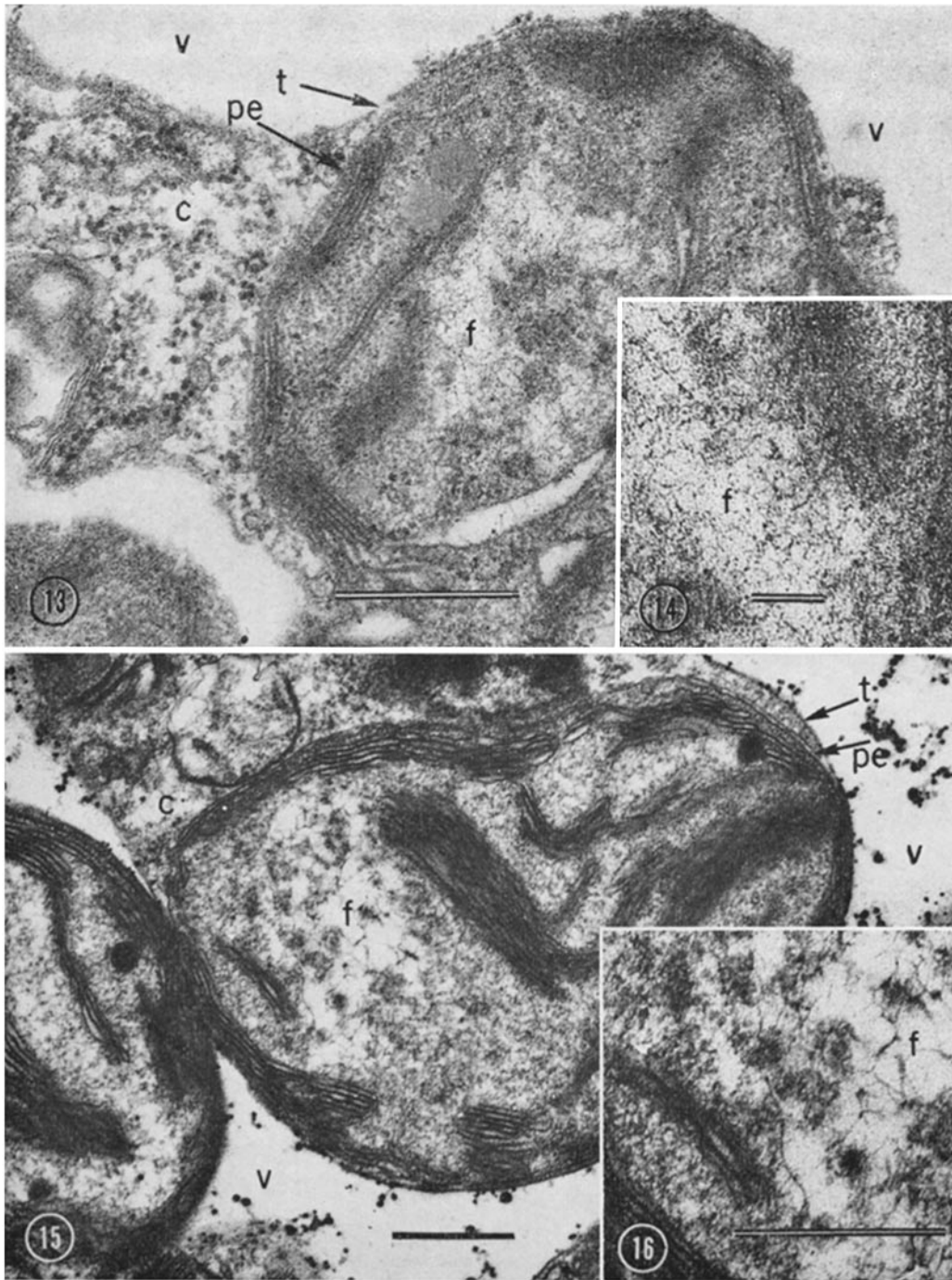
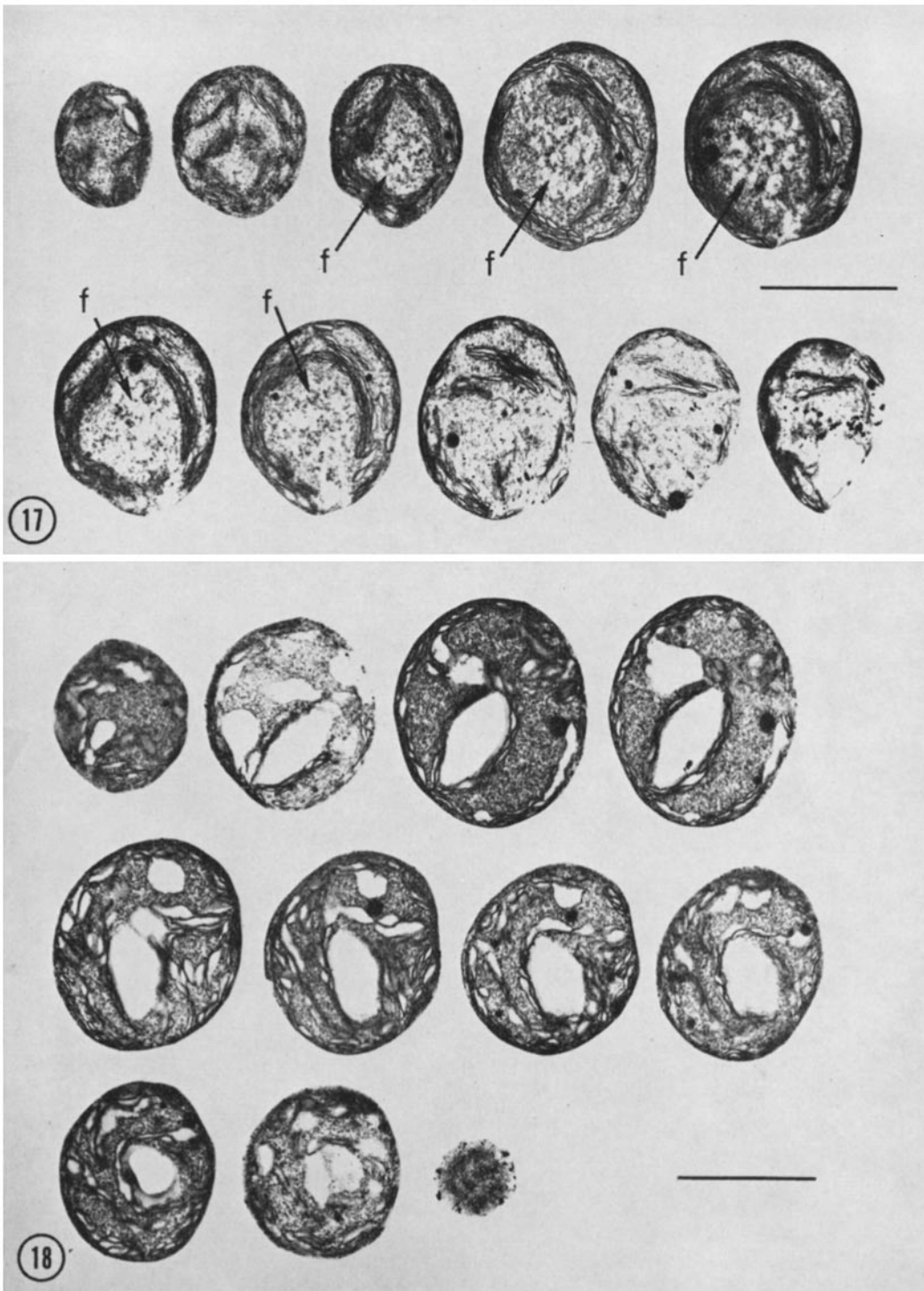


FIGURE 12 Distribution of silver grains over plastids of *Acetabularia mediterranea* following incorporation of actinomycin D-³H and radioautography. Left, preparation was treated with DNase prior to application of the emulsion. Right, identical preparation except that DNase was omitted. Shaded area indicates significant incorporation (i.e., the increase over the DNase-treated plastids). Shaded region comprises 33% of total area.



FIGURES 13-16 Thin sections of glutaraldehyde-fixed *Acetabularia mediterranea* plastids. Figs. 13 and 14 Veronal-acetate buffer; Figs. 15 and 16 phosphate-seawater buffer. Figs. 14 and 16 fibrillar region. *t*, tonoplast; *v*, vacuole; *pe*, plastid envelope; *f*, fibrillar area; *c*, cytoplasm. Bars in Figs. 13, 15, and 16 = 0.5 μ ; bar in Fig. 14 = 0.1 μ . Fig. 13, $\times 53,000$; Fig. 14, $\times 100,000$; Fig. 15, $\times 35,000$; Fig. 16, $\times 60,000$.



FIGURES 17 and 18 Assembled serial sections of glutaraldehyde-fixed plastids of *Acetabularia mediterranea*. Fig. 17 shows a series with typical fibrillar DNA-containing area in five sections. *f*, fibrillar area. Bar = 1.5μ . $\times 15,000$. Fig. 18 shows a series lacking a visible fibrillar DNA-containing area. Bar = 1.5μ . $\times 15,000$.

TABLE III
Distribution of DNA-Containing Areas in Serially-
Sectioned Plastids

Species	No. plastid series showing DNA-containing areas	Total No. plastid series	% of plastids with DNA areas
<i>Acetabularia mediterranea</i>	4	18	22
5 mm stalk	20	107	19

tect very small quantities of it, but the use of the Kleinschmidt spreading method to visualize individual DNA strands makes it possible to quantify and to achieve high sensitivity.

Electron Microscopy—Spreading with Osmotic Shock

Acetabularia plastids were found to be highly susceptible to osmotic shock, despite a report to the contrary by Goffeau (9). When spread on a water surface, the plastid membrane system was seen as a single layer with very few overlapping regions. Since the total cytoplasmic content of a cell was spread, the shadowed preparations contained spread mitochondria and other membranous components, as well as plastids. However, the membranes released from a plastid were distinctive and covered such a large area (about $40 \mu^2$) that plastids were unlikely to be confused with mitochondria. Even if a spread plastid were contaminated with mitochondria, it is unlikely that DNA contributed by the latter would seriously affect the results, since none of the other DNA-detecting techniques indicated comparable concentrations of DNA within mitochondria. Associated with plastid membranes, in some cases, were aggregates of fibrils and particles that showed a remarkable similarity to the fibrillar regions seen in sections of plastids (Fig. 19). When DNase-free Pronase was included in the spreading mixture, the aggregates were more widely dispersed, permitting rough estimates of the total strand length (Fig. 20). After brief treatment with DNase, a rapid disintegration of the filaments was observed (Fig. 21). However, no fibrillar component could be detected in the majority of spread plastids. The proportion of

plastids in which fibrils were observed was calculated from four groups of experiments. The results are shown in Table IV. The total fibril length per plastid could be estimated in only a few cases, due to the highly tangled aspect of the strands. These measurements are shown in Fig. 22. Although the Kleinschmidt technique is potentially very sensitive, enabling DNA strands a fraction of a micron in length to be seen and measured, when applied to plastids there is the probability that at least a portion of any DNA released will be obscured by membranes. It is, therefore, difficult to establish a lower limit to the sensitivity of the method in this case.

The greatest total strand length recorded from a single *Acetabularia* plastid was about 1000μ . It is inconceivable that this amount of DNA was present in a similar form in all plastids since, when spread, it would occupy far too great an area to be obscured in 75% of plastids. Moreover, even if only a few microns of DNA were present, it is extremely unlikely that the DNA would be completely hidden in over 120 instances. However, we cannot exclude the possibility that some DNA is present in 75% of plastids, but in a state in which it is not liberated by the spreading process.

From the few length measurements, it can be seen that the average strand length of DNA in the 25% of plastids with detectable DNA is of the order of hundreds of microns and probably lies between 200 and 400μ . When this is averaged over the total plastid population, the range is between 50 and 100μ . In a fluorometric analysis of the amount of DNA in *A. mediterranea* plastids, Gibor and Izawa (8) found 10^{-16} g DNA/plastid, which is equivalent to approximately 30μ of double-stranded DNA (35). Although our estimate of 50– 100μ is two- to threefold greater than this value, when we consider the errors involved in both methods such differences may not be significant. Recently, Werz and Kellner (33) have reported that extracted *A. mediterranea* plastid DNA appears in spread preparations as aggregates about 30μ in length, which could be taken to support the estimate of Gibor and Izawa. There is, however, no evidence that each of the aggregates of tangled DNA strands observed by Werz and Kellner (33) originated from a single plastid, and data argue against the conclusion that each plastid contains a single 30μ length of DNA.

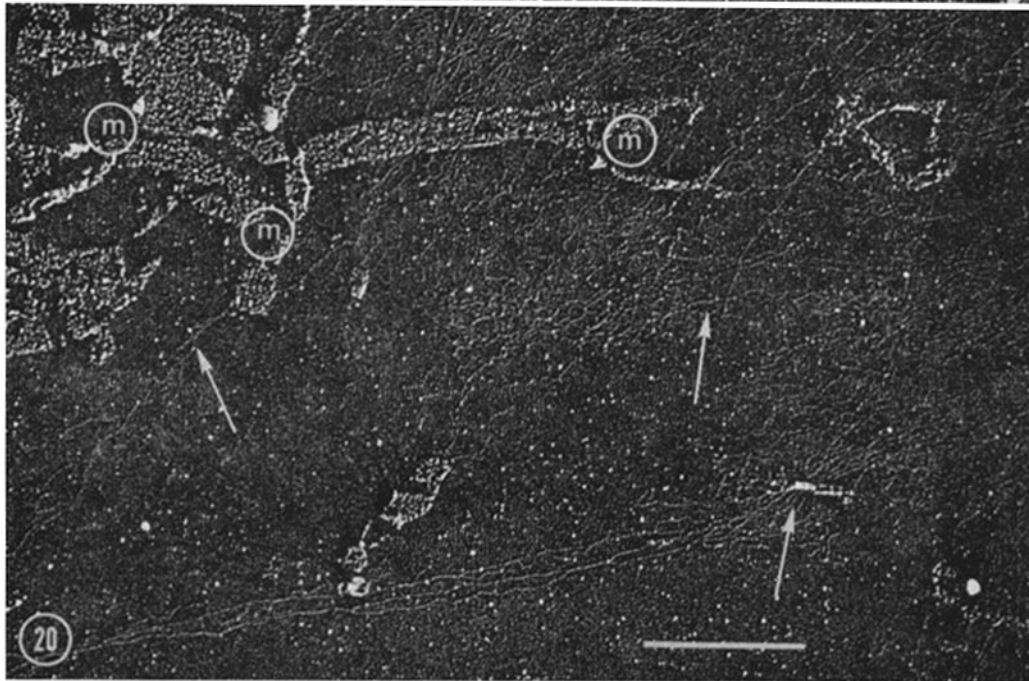
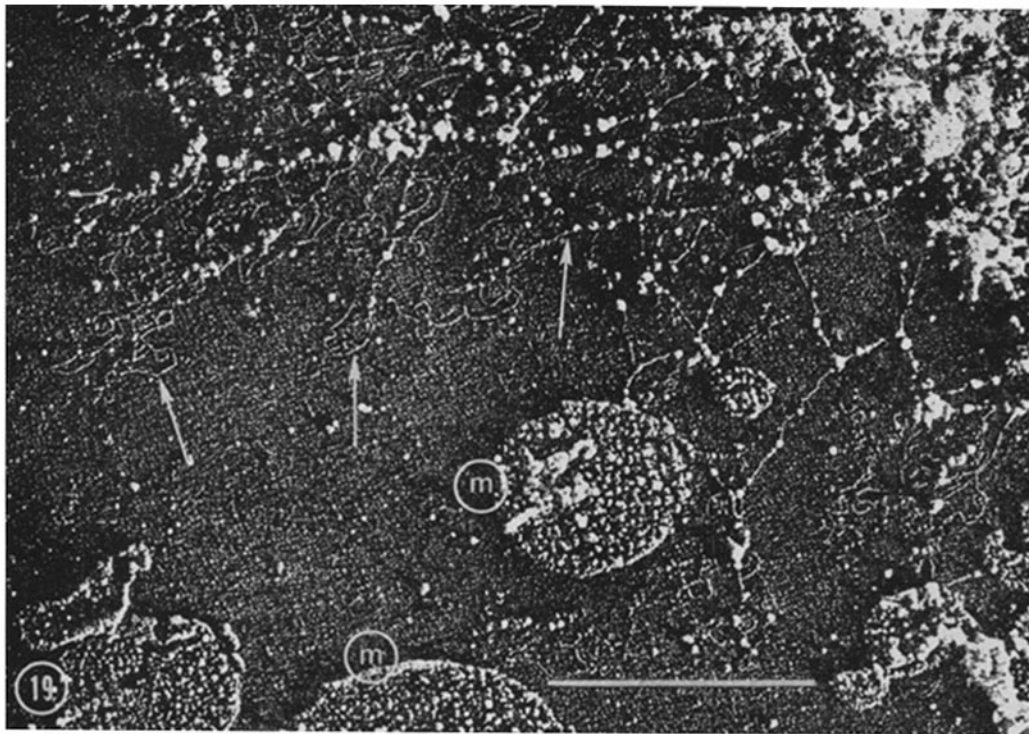


Figure 19 Portion of the DNA-containing area associated with a disrupted plastid of *Acetabularia mediterranea*. Arrows indicate DNA strands. *m*, plastid membranes. Bar = 1 μ . $\times 35,000$.

FIGURE 20 Portion of the DNA-containing area associated with a disrupted plastid of *Acetabularia mediterranea*. Pronase was included in the spreading mixture, giving an increased separation of DNA strands compared with Fig. 19. Arrows indicate DNA strands. *m*, plastid membranes. Bar = 1 μ . $\times 21,000$.

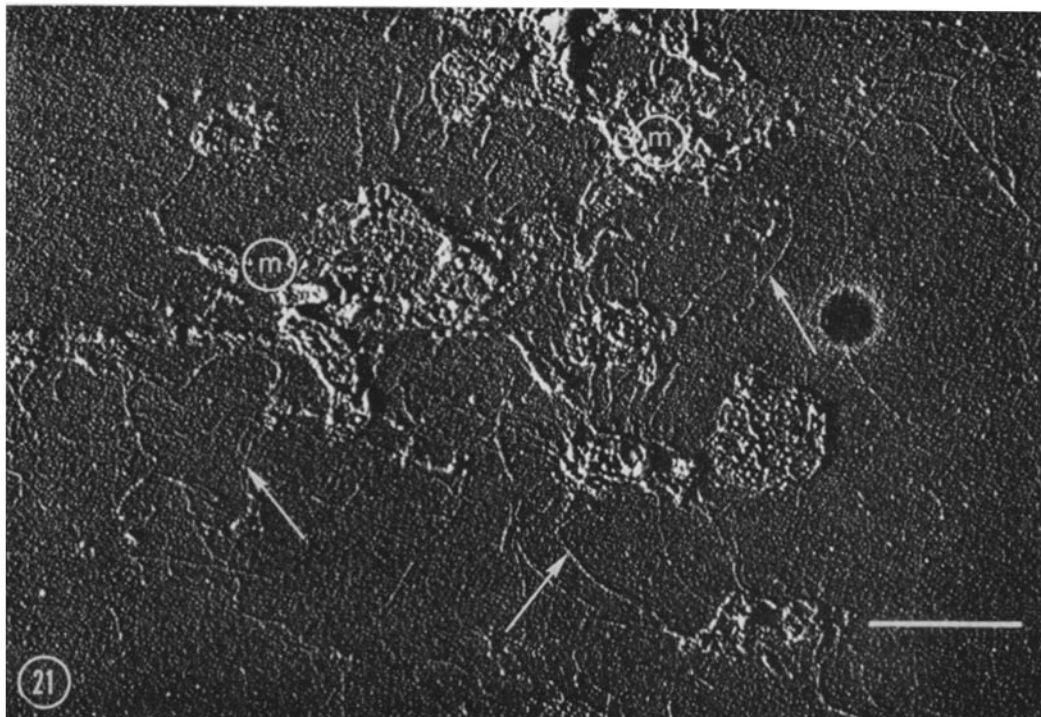


FIGURE 21 Portion of the DNA-containing area associated with a disrupted plastid of *Acetabularia mediterranea*. After spreading, the specimen was treated with DNase for 2 min, resulting in breakage of the DNA strands. Arrows indicate DNA strands. *m*, plastid membranes. Bar = 1 μ . $\times 20,000$.

TABLE IV
Distribution of DNA Strands from Spread Plastids

Species	No. spread plastids with DNA strands	Total No. spread plastids	% of plastids with DNA strands
<i>Acetabularia mediterranea</i>	7	28	25
	9	43	21
	16	50	32
	15	50	30
		Mean	27

DISCUSSION

The four techniques used here give essentially the same result: DNA occurs in varying amounts in 20–35% of *Acetabularia* plastids but is undetectable in the remaining 65–80%. Although we have not proved that the localization involved the same 20–35% of plastids in each method, there is considerable circumstantial evidence that this is

the case. For example, in size and shape range the DNA areas seen after acridine orange staining are similar to those seen in thin sections; the appearance of the spread DNA recalls that of DNA-containing areas in thin sections (compare Figs. 16 and 19). Also, DNase specificity was demonstrated in three of the techniques.

The main limitations and advantages of each method we have used are listed in Table V. It can be seen that although each method has major disadvantages no single problem is common to all four. For example, the effect of microorganism contamination even in apparently sterile cultures has been demonstrated by Green et al. (11), but the possibility that microorganisms were contributing to the results can be discounted absolutely in the case of the serial sections. If the DNA were widely dispersed throughout the stroma, the acridine orange staining, and possibly serial sections, might fail to detect it, but actinomycin D labeling and electron microscopy of spread plastids would not be affected.

Thus, the combined results argue that the

observations are valid and cannot be attributed to external factors. There are nevertheless at least three ways in which the data can be interpreted:

(a) All *Acetabularia* plastids contain the same amount of DNA (of the order of hundreds of microns), some of it accessible to the localizing techniques used, and the remainder inaccessible. The ratio of accessible to inaccessible DNA would vary in 20–35% of plastids, while in the other 65–80% all the DNA would be inaccessible. This interpretation seems most unlikely since, even if the “inaccessible” DNA were within the membrane system, after fixation and ethanol treatment, the membranes should be permeable to acridine orange and actinomycin D.

the true genetic coding capacity. The apparent conflict between Gibor and Izawa's estimation of the quantity of DNA/plastid (8) and our DNA length measurements has already been discussed (see page 369).

The synthetic capacities of *Acetabularia* plastids have been well documented in vivo and in vitro by incorporation studies of DNA, RNA, and protein precursors (4, 10, 14, 15, 29, 30). These observations suggest that the plastids possess their own genetic information and the means of transcribing and replicating it. Our data, suggesting that the quantity of DNA/plastid is variable, seem at first incompatible with such a system, unless the assumption is made that it is

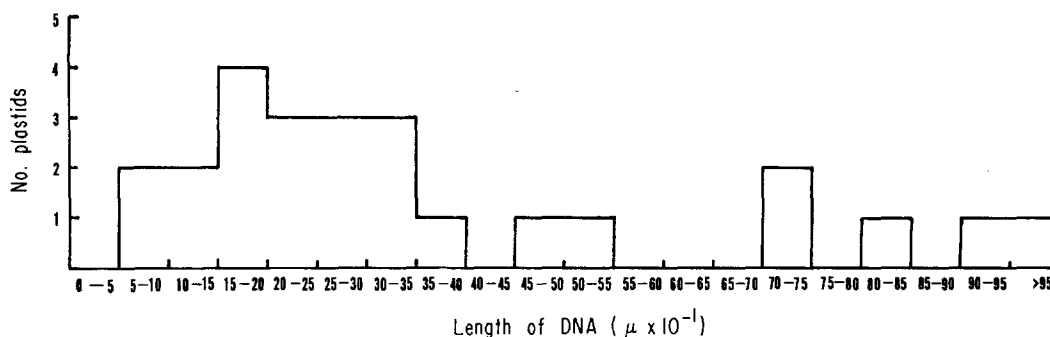


FIGURE 22 Length distribution of DNA strands associated with individual disrupted plastids. Only plastids with attached DNA are included.

(b) All *Acetabularia* plastids contain DNA; in 20–35% it exists as a variable amount, condensed into one or two regions of stroma. In the remainder, an unspecified amount, too small to be detected by actinomycin D- ^3H labeling and not released during spreading, would be present. As in (a), an inaccessible form of DNA has to be invoked.

(c) Only 20–35% of plastids contain DNA, and in these the amount is variable ranging from 50 to 1000 μ .

With the methods presently available, there seems little chance of deciding between the two latter interpretations.

A variable amount of DNA/plastid is involved in interpretations (b) and (c) above; and besides the theoretical considerations this raises, there are also practical implications. Estimates of the amount of DNA/plastid based on extraction and assay on a mass scale cannot allow for variability, and the average value obtained may not reflect

the number of copies of a genome of a fixed but very small size that varies. Although methods based on shearing and reannealing of the DNA (5) are available for estimating the number of copies, the quantities of DNA necessary are prohibitive at present in the case of *Acetabularia*.

If the quantity of DNA/plastid is variable and if all of this DNA is biochemically active, then it should be possible to detect differences in DNA and RNA synthesis between plastids. An radioautographic study by Shephard (30) of the incorporation of thymidine and uridine into *A. mediterranea* plastids showed that differences among plastids in the ability to synthesize DNA and RNA were indeed present. Shephard's results, presented in histogram form in Figs. 23 and 24, show a striking resemblance to our radioautographic data (see Fig. 12) and may be interpreted in a similar fashion (see page 364). Our estimations from these results of the number of plastids with above-background label after

TABLE V
Limitations and Advantages of the DNA Localization Techniques

Technique	Limitations	Advantages
Acridine orange staining	DNA must be present in sufficient quantity and in a sufficiently condensed state. Contamination with other organelles or microorganisms cannot be completely excluded.	Rapid method. Many plastids can be scored.
Actinomycin D- ³ H labeling	Contamination cannot be excluded.	Sensitivity can be increased by longer exposure time. Does not depend on DNA being in a condensed state.
Electron microscopy of sections	Few plastids can be scored. Depends on DNA fibrils being visible.	Highly sensitive. Contamination can be excluded.
Electron microscopy of spread plastids	Obscuring effect of membranes reduces sensitivity. Contamination cannot be absolutely excluded.	Does not depend on DNA being in a condensed state. Allows quantitation.

thymidine-³H and uridine-³H incorporation are 28% and 36%, respectively, and are in good agreement with the 20–35% values for the proportion of plastids in which DNA could be detected.² We have repeated these labeling experiments, and the preliminary results of uridine-³H incorporation into plastid RNA are fully in agreement with those of Shephard. Thus, it

² See Note 2 Added in Proof.

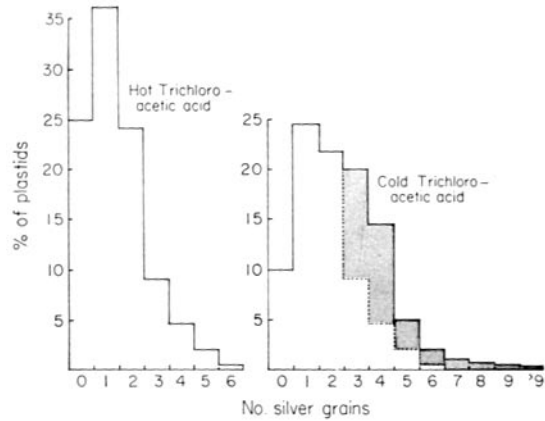


FIGURE 23 Distribution of silver grains over plastids of *Acetabularia mediterranea* following in vivo incorporation of thymidine-³H. Data taken from Shephard (30). Left, after treatment with hot trichloroacetic acid followed by cold trichloroacetic acid. Right, after cold trichloroacetic acid only. The shaded area indicates significant incorporation (i.e., the increase over the hot trichloroacetic acid-treated plastids). Shaded region comprises 28% of total area.

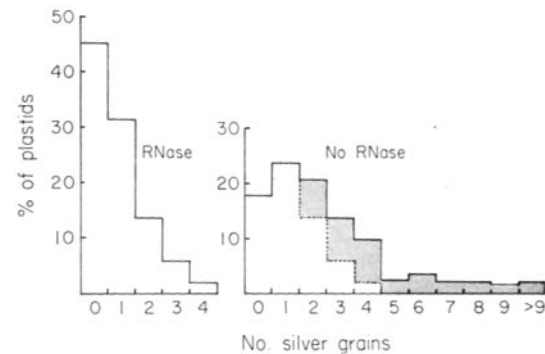


FIGURE 24 Distribution of silver grains over plastids of *Acetabularia mediterranea* following in vivo incorporation of uridine-³H. Data taken from Shephard (30). Left, preparation treated with RNase. Right, identical preparation except that RNase was omitted. Both preparations were treated with cold trichloroacetic acid before application of the emulsion. The shaded area indicates significant incorporation (i.e., the increase over the RNase-treated plastids). Shaded region comprises 36% of the total area.

would seem that the amount of DNA present in *Acetabularia* plastids is roughly proportional to the amount of replication and transcription, and that the large quantity of DNA present in some plastids is not inactive.

If interpretation (c) above is considered, the

following problem arises: how can plastids with no DNA at all operate within a semiautonomous system? It could be speculated that, in this case, division of plastids was unequal, one plastid retaining all the DNA; the DNA-less daughter would be capable of photosynthesis for a while, but supplies of messenger RNA would eventually be exhausted and the plastid would "die." On the other hand, reports of dynamic interactions between organelles during cyclosis in plant cells (34) suggest that the plastids of a cell might be envisaged as a single unit, existing at a given time in an arbitrarily divided state. In the case of *Acetabularia* the plastids with no DNA would be continually making up any deficiency by fusion with DNA-containing ones. Although we have been unable to obtain any evidence for fusion of plastids by direct observation of living cells, time-course studies of uridine-³H incorporation should be able to resolve this question. (Our experiments on uridine-³H uptake, like those of Shephard [30], have so far been limited to 4 hr incorporation time.)

In previous work there is little evidence that can be cited to support or contradict the possibility that DNA-less plastids may exist in plants

with more than one plastid/cell. However, in Swiss chard, all plastids were shown to stain with azure B (18). Target-theory analysis of UV-induced bleaching in *Euglena* indicated that about 30 sites were involved in a system with about 10 plastids, the inference being that there were three receptive regions of nucleoprotein/plastid (20, 28). On the other hand, it may be pertinent to note that in similar studies on "petite" induction in yeast mitochondria, Maroudas and Wilkie (21) concluded that the number of genetically effective copies of mitochondrial DNA in aerobic cells appeared to be far smaller than the number of mitochondria.

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Note 1 Added in Proof: A more satisfactory method of determining whether data such as those of Fig. 12 (no DNase) comprise two populations is to plot

$$\ln \frac{p}{100 - p}$$

[where p is the cumulative percentage] against number of grains. A single Gaussian distribution gives a straight line, whereas two overlapping distributions give a sigmoid curve in which the position of the point of inflection is determined by the relative sizes of the two populations. By such an analysis, the data of Fig. 12 (no DNase) are shown to consist of two populations, one identical in mean and spread to the (DNase) population, the second with a higher mean. Referring to Table II, if the percentage of plastids with significant label are calculated by this method, the new values are [reading from the top] 18% ± 5%, 32% ± 10%, 28% ± 5%, 27% ± 5%, respectively.

Note 2 Added in Proof: Analysis of Shephard's uridine data into Gaussian distribution (see *Note 1* above) indicates two populations in the control (no RNase) data, one with a mean of about seven grains per plastid and comprising 10% of the total, and a second, similar to but with a mean which is one grain per plastid higher than that of the RNase background. In the thymidine data, more than one population is indicated, but the lack of a definite sigmoid curve precludes further analysis.