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CHAPMAN, Carl Joseph, 1939—
DNA IN THE CHLOROPLASTS OF ACETABULARIA MEDITERRANE A.

University of New Hampshire, Ph.D., 1965
Botany

University Microfilms, Inc., Ann Arbor, Michigan
DNA IN THE CHLOROPLASTS OF

ACETABULARIA MEDITERRANEAE

BY

CARL JOSEPH CHAPMAN

B. S., University of New Hampshire, 1961

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School
Department of Botany

June, 1965
This thesis has been examined and approved.

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Acknowledgments

I am grateful to Dr. Richard W. Schreiber for his guidance during the planning and research phases of this thesis and for his patience in reviewing the manuscript.

I wish to thank Dr. Noel A. Nugent for his assistance in carrying out some of the technical phases of the research and in culturing the algae.

I wish to thank Dr. Konrad Keck, formerly of Johns Hopkins University, now at the University of Arizona, for his help with the initial phases of the culture method and for supplying the starting cultures.
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INTRODUCTION

For several years there has been speculation about the possible autonomous nature of chloroplasts. A current theory holds that chloroplasts, as we know them, evolved from a blue-green alga-like cell living symbiotically within an ancient protozoan. If such were the case, one might expect to find at least remnants of the various metabolic processes characteristic of an autonomous cell.

This study is designed to investigate the presence of deoxyribonucleic acid (DNA) in the chloroplasts of *Acetabularia mediterranea*. To demonstrate the presence of DNA in chloroplasts, it is of the utmost importance to be able to eliminate completely any possibility of nuclear DNA interfering with the determination. *Acetabularia* is the ideal organism for these studies: it can easily be enucleated, its cells will live for several months in this enucleated state, and its size and growth habit make it ideally suited for the various manipulations required in the experiments.

One group of investigators has looked for DNA in the chloroplasts of *Acetabularia mediterranea* using a direct extraction of DNA from the chloroplasts. Their results were low enough to raise questions as to the possibility of bacterial contamination or at least incomplete extraction. Another group used the incorporation of tritiated thymidine with radioautography. Their results seemed inconclusive as they reported "uniform cytoplasmic labeling."

Investigators extracting DNA from chloroplasts of other plant species have all failed to demonstrate complete freedom from nuclear
contamination in their chloroplast preparations. The sucrose density
gradient method of chloroplast "purification" that is almost universally
used has been proved to be ineffective in removing all nuclear con-
taminants.

Nucleophyllic stains have produced varied results which are far
from being conclusive.

There is a great deal of indirect evidence that would lead one to
believe that DNA is present in the chloroplasts. For example, it has
been recognized for some time that chloroplasts are capable of growing
and dividing independently of the growth and division of the cell. In
Acetabularia, the chloroplasts grow and divide in enucleated cells.
Chloroplasts are also known to mutate without nuclear intervention.

There has been a considerable amount of work done on the bleach­
ing of chloroplasts, particularly in Euglena, by ultraviolet light. It
has been found that while exposure of the whole cell to sublethal doses
of UV light causes complete bleaching of the chloroplasts, microbeam
irradiation of only the nucleus produces no bleaching. Since the action
spectrum of the UV bleaching suggests a nucleoprotein target, it is
likely that it is the action on extranuclear DNA that causes the bleaching.

While thymidine is readily incorporated into DNA and is usually
used in tracer studies concerning DNA, the free base thymine is not well
utilized. Certain bacterial strains require thymine for their growth.
It has been noted that thymine is incorporated into a "nucleic acid
fraction" in Acetabularia.

It is the purpose of this thesis to prove conclusively the
presence of DNA in the chloroplasts of Acetabularia mediterranea and to
determine some of the metabolic characteristics of this DNA.
The first statement of the theory of the endosymbiotic relationship of the blue-green algae in protozoa evolving into the chloroplast was put forth by Mereschowski in 1905. This was followed by a similar statement by Famintzin in 1907. Rhodes (1946) states that, "The belief that plastids of higher plants are autonomous rests chiefly upon the non-Mendelian heredity of certain plastid variegations, where it appears that two distinct and separable kinds of plastids are involved." Weier and Stocking (1952a) review chloroplast inheritance. A more recent article by Granik (1961) also reviews this subject.

Many investigators maintain that there is no DNA in chloroplasts. Among them, Holden (1952) found 36.9% of the total phosphorus to be in the nucleic acid fraction of the chloroplast fraction but was unable to demonstrate unequivocally the presence of DNA. Using various stains such as Feulgen, methyl green, pyronin, and azure B, Littau (1958) found no DNA in chloroplasts of any of the several species that he studied. Ruppel (1964) hydrolyzed chloroplasts of *Allium porrum* and *Antirrhinum majus* with concentrated formic acid. He found no thymine among the other bases and thus concluded that no DNA occurred in chloroplasts from adult leaves of higher plants.

Chiba (1951) found that staining with methyl green and pyronin revealed DNA and RNA in chloroplasts of *Salaginella Savatieri*, *Tradescantia fluminensis*, and *Rhoeo discolor*. Spikermann (1957) found DNA in proplastids of *Chlorophytum comosum* and *Helianthus tuberosus* after staining with Feulgen. He found that after $\frac{1}{2}$ hour treatment with deoxyribonuclease (DNase) the nuclei of Helianthus no longer stained with
Feulgen. Primary grana stained even after 1, 2, 3, and 4 hour treatments. Five hour treatments were required to remove stainability.

Metzner (1952) and Ris and Plaut (1962) also have demonstrated DNA in chloroplasts with various staining techniques. The latter paper also presents electron micrographs of chloroplasts of *Chlamydomonas moewussi* showing 25 Å microfibrils in the areas known to take up the stains. These microfibrils were identified as DNA molecules on the basis of their location, morphology, and sensitivity to DNase digestion.

Stocking and Gifford (1959) showed incorporation of $^{3}H$-thymidine into chloroplasts of Spirogyra. After 27 hours of incubation of cells in 10 μC/ml $^{3}H$-thymidine, between 81 and 91% of the radioactivity was found to be associated with the chloroplasts. After 95 hours, 86-94% was found with the chloroplasts. Radioautography was used to detect the location of the radioactivity. They pointed out some of the dangers of considering thymidine as a precursor exclusively for DNA, but they reported no DNase controls.

Brachet (1959) has reported that *Acetabularia mediterranea* cells incubated with $^{3}H$-thymidine for 30 minutes to 3 hours showed "uniform cytoplasmic label." He mentioned that one of his coworkers had succeeded in removing this label with DNase, but this has never been published. This paper was cited in a later report, Baltus and Brachet (1963), as "proof" that Acetabularia chloroplasts incorporate $^{3}H$-thymidine into DNA. In this latter note (1963), a fluorometric method was used to determine the amount of DNA in various portions of Acetabularia cells. The results are given in the following table.
DNA in Various Portions of Acetabularia Cells

Cap  0.58, 0.60, 0.70, 0.015 \( \mu g \) DNA
Cyst  4.2 \( \times 10^{-9} \), 5.0 \( \times 10^{-9} \) \( \mu g \) DNA
Chloroplasts of a single alga  0.0059, 0.0037 \( \mu g \) DNA

Sagan and Scher (1962) reported \( H^3 \)-thymidine incorporation into cytoplasm of normal and dark grown Euglena cells but not into streptomycin bleached cells. Acid-alcohol insoluble label was DNase removable.

Cooper and Loring (1957), using phosphorous content as a guide, found DNA to comprise 0.7% of the weight of acid-washed tobacco chloroplasts. These chloroplasts were isolated by differential centrifugation in sucrose.

Weier and Stocking (1952b) conducted cytological studies on young tobacco leaf homogenates by 1.) Feulgen, 2.) toluidine blue staining of osmium fume-fixed smears, and 3.) methyl green staining. They found that in smears from the fresh, unstained final pellet of a typical sucrose separation, neither nuclei nor nuclear threads were apparent. Feulgen staining brought into evidence numerous slender, positive threads and some nuclei.

The following table appeared in a paper by Chiba and Sugahara (1957).

<table>
<thead>
<tr>
<th>Analysis</th>
<th>mg/Chloroplast</th>
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<tr>
<td></td>
<td>Spinach</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>7.0 ( \times 10^{-10} )</td>
</tr>
<tr>
<td>Dry weight</td>
<td>7.7 ( \times 10^{-9} )</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>6.8 ( \times 10^{-10} )</td>
</tr>
<tr>
<td>RNA Phosphorous</td>
<td>2.9-4.5 ( \times 10^{-12} )</td>
</tr>
<tr>
<td>DNA Phosphorous</td>
<td>3.8-6.9 ( \times 10^{-12} )</td>
</tr>
</tbody>
</table>
The ratio of nuclear contaminants to chloroplasts in the preparations used in these analyses was reported to have been reduced to about 1:600 in both the spinach and tobacco. Total nucleic acid content per chloroplast was found to be 6-10 times greater than that reported by Jagendorf and Wildman (1954).

Pollard (1963) showed that spinach chloroplasts purified by density gradient techniques contained 3-5 \( \mu \)g of DNA per mg of chloroplast protein. Crude nuclear particles contained 30-50 \( \mu \)g/mg protein.

Sager and Ishida (1963), also using sucrose density techniques for isolating chloroplasts, found 1.2 \( \times 10^{-11} \) mg of DNA per chloroplast of Chlamydomonas.

Chun et al (1963) found chloroplast DNA to be equal to 1-10\% of the whole cell's DNA in Chlamydomonas, Chlorella, spinach, and beet. Ultracentrifugal analysis of the DNA from chloroplast preparations showed two forms. The alpha component was found to be by far the major component in analyses of the whole cell's DNA. In chloroplast preparations purified by a standard density gradient procedure, this nuclear component still comprised 55\% of the DNA. This again points out the dangers of basing any quantitative estimations of DNA content of chloroplasts that have been isolated by density gradient techniques.

Leff et al (1963) found satellite bands in ultracentrifugal analysis of DNA from normal green Euglena cells. These bands were absent in DNA from aplastidic cells. It was concluded that these bands were attributable to chloroplast DNA.

Gibor and Izawa (1963) investigated the DNA of Acetabularia chloroplasts with the idea of making quantitative estimations of DNA in plastids. Here for the first time was a determination in which nuclear
contamination was eliminated. Using fluorometric procedures, they estimated a value of $1 \times 10^{-16}$ g of DNA per plastid. With values this low, one must be very careful of bacterial contamination. Their cells were grown in the Erdschreiber medium with additions of $K_2HPO_4$, glucose, and tryptose to accelerate the growth of any contaminants. Any culture flasks exhibiting turbidity were immediately discarded. Their cells were also taken through a complicated antibiotic solution before use.

Wollgiehn and Mothes (1963, 1964) studied young (growing) and old (nongrowing), sprouted and unsprouted leaves of *Nicotiana rustica* using $H_3$-thymidine and radioautography. They found label to be taken up by chloroplasts in the growing leaves (sprouted and unsprouted) but not by those in nongrowing leaves. The label was taken up by an acid-insoluble, DNase-hydrolyzable substance.

Olszewska and Mikulska (1964) studied cells of white zones (I), pale green zones (II), and green zones (III) of *Clivia miniata* and *Bilbergia sp.* They found $H_3$-thymidine to be incorporated into plastids in zones II and III but not in zone I. Similar results were obtained with $H_3$-uridine. However, $H_3$-phenylalanine was incorporated mostly in zone I.

Brawerman and Eisenstadt (1964) studied DNA from the chloroplasts of *Euglena gracilis* and found a major component with a density of 1.684 in addition to the nuclear component with a density of 1.708. Partial lysis of the chloroplasts was found to result in removal of the nuclear component. A component with a density of 1.692 was also associated with the chloroplast preparations. The chloroplast DNA was found to melt at 78-80 °C while the nuclear DNA melts at 89-91 °C. The guanine and cytosine content of the chloroplast DNA is about 25 mole percent. Some
5-methylcytosine was found in the nuclear material. The amount of DNA per chloroplast was found to be approximately equal to that of an *Escherichia coli* cell or $0.9-1.1 \times 10^{-14}$ g/cell.

Considerable work has been done with bleaching of Euglena and other plant cells with ultraviolet light. Among those investigating this process were Lyman, *et al* (1959), Blakely and Chessin (1959), Lyman, *et al* (1961), Schiff, *et al* (1961a), Schiff, *et al* (1961b), and Gibor and Granik (1962). Lyman, *et al* (1961) found that the action spectrum for the UV inactivation of chloroplast formation shows peaks at 260 and 280 m\(\mu\), implicating nucleoprotein as the probable site of UV action. They proposed that the UV sensitive site was cytoplasmic. Their evidence leads to the hypothesis that there are self-reproducing cytoplasmic entities in Euglena which contain nucleoprotein and which are responsible for the development of chloroplasts.

Gibor and Granik (1962) confirmed these theories by irradiating whole Euglena cells with sublethal doses of UV light. This treatment was found to cause bleaching and slight inhibition of cell division. Microbeam irradiation of only the nucleus produced no bleaching. This was considered as evidence for the localization in the cytoplasm of genetic factors responsible for the development of chloroplasts.

Benkers and Berends (1960), Marmur and Grussman (1961), and Benkers and Berends (1961) studied the effect of UV light on DNA. They concluded that the inactivation of DNA was due to a formation of thymine dimers thus linking DNA strands. It was also found that these dimers were broken by an enzyme which is activated by light. This was consistent with the findings of other investigators, including Schiff, *et al* (1961a) who found that chloroplast bleaching was photoreversible with visible light.
The many early reports of DNA in chloroplasts are reviewed and criticized by Granik (1961) and Smillie (1963).

Many investigators have studied the incorporation of labeled nucleic acid precursors into DNA. A few that are pertinent to this study include Plantel and Schoenheimer (1944), McQuade, et al (1956), Freidkin, et al (1956), Amano, et al (1959), and Magasanik (1962).
MATERIALS AND METHODS

Experimental organism. Acetabularia mediterranea is a single-celled green alga (division Chlorophyta) in the family Dasycladaceae. The cell consists of a rhizoidal area containing the single nucleus, a stalk that may reach 2-6 cm in length at maturity, and a cap that is formed at maturity. For a complete discussion of the organism, see Hammerling (1931, 1944), Brachet (1961), and Keck (1964).

Acetabularia cultures. Dark grown stock cultures of Acetabularia mediterranea were obtained through the generosity of Dr. Konrad Keck at Johns Hopkins University, Baltimore, Maryland.* To obtain working cultures from these stock cultures, it was necessary to place a few drops of solution containing the young plants into 150 ml of growth medium in pyrex crystallizing dishes, 50 X 90 mm, covered with 100 mm petri dish covers.

The usual growth medium for Acetabularia is Erdschreiber medium which has the following composition:

- Sterile sea water 5000 ml
- Earth extract 70 ml
- \( \text{NaNO}_2 - \text{Na}_2\text{HPO}_4 \) concentrate 30 ml
- Sterile distilled water 200 ml

Sea water was sterilized by boiling for three minutes at normal atmospheric pressure. Autoclaving of sea water produces an undesirable precipitate. The earth extract was prepared by autoclaving 50 g of rich humus soil for 20 minutes at 120 °C in 500 ml of sea water. The hot *Dr. Keck's present address is the University of Arizona, Tucson, Arizona.
suspension was immediately filtered through a glass wool filter and then through a fluted filter (Schleicher and Schuell, #588, or equivalent). The filtrate was reautoclaved and stored in a refrigerator. The salt concentrate consisted of 20.0 g of NaNO₃ and 4.0 g of Na₂HPO₄ in 1000 ml of distilled water. The salt solution should be sterilized by autoclaving and stored in the refrigerator. The distilled water should be autoclaved or boiled.

Artificial sea water (Rila Seven Seas Marine Mix, Utility Chemical Co., 145 Peel Street, Paterson, New Jersey) is a completely satisfactory substitute for Erdschreiber medium with the added advantage of being autoclaveable. When the artificial sea water is used, the composition of the growth medium is as follows:

Artificial sea water mix 199.5 g
NaNO₃- Na₂HPO₄ concentrate 10.0 ml
Sterile distilled water 5000 ml

Under ordinary culture conditions, it was necessary to brush these primary cultures every other day with a soft camel’s hair brush to prevent the young cells from attaching themselves to the glass. It was found that a coating of teflon on the bottoms of the culture dishes prevented this sticking and eliminated the need for brushing (Schreiber, et al, 1964). The teflon coatings combined with the Rila Marine Mix greatly cut down the chances for contamination.

To insure sterility, all cells were treated prior to use in experiments by a method suggested by Dr. Keck. This involved immersion of the cells for 48 hours in a medium containing Penicillin and Streptomycin. The antibiotic solution contained 100,000 units of Penicillin G and 100 mg of Streptomycin sulfate per ml. To each 100 ml of culture medium was added 0.05 ml of the antibiotic solution. The cells were rinsed in normal growth medium following this treatment.
The primary cultures were thinned out as soon as they could easily be handled (2-3 weeks). The final concentration in the working cultures was 50-60 cells per dish. Cells in these working cultures attained usable size in 1-2 months.

See Figure 1 for the growth pattern of Acetabularia.

Preparation of Acetabularia cells for radioautography. Cells 4-5 cm long were chosen for radioautography. The cells were placed in the above antibiotic solution for 48 hours, then washed and placed in fresh medium to be cut. The cutting was accomplished with DeWecker iridectomy scissors with short, pointed blades (Arista Surgical Company, 67 Lexington Avenue, New York 10, New York). For the purposes of these experiments, the cells were cut approximately in half (Figure 2) and left undisturbed for at least four days. Seventy-five to eighty percent of the enucleated portions survived and nearly 100% of the nucleated portions survived. Dr. Keck (personal communication) suggested various methods such as a two-day dark treatment prior to cutting, or tying a fine thread around the stalk to prevent loss of cytoplasm, as means of increasing survival after cutting. The former treatment was tried and seemed to help some. The latter treatment was not tried. Dead cells are distinguished by a clumping of the cytoplasm producing a spotty appearance (Figure 1).

Following the recovery period, the surviving cells were placed in media with tritiated thymidine or tritiated thymine. The times of incorporation are noted in the results section. The $\text{H}^3$-thymidine and $\text{H}^3$-thymine (1mC/ml, Schwarz Bioreserch, Inc., Orangeburg, New York, and New England Nuclear Corp., 575 Albany St., Boston 18, Massachusetts) were used in the concentration of 2.5 $\mu$C/ml. After the period of incubation
with the label, the cells were placed in the normal growth medium for one hour to remove any excess label from the outside of the cell.

**Preparation of the slides for radioautography.** Glass microscope slides were thoroughly cleaned in chromic acid cleaning solution, rinsed, dried, and coated with a gelatin solution prepared by dissolving 5 g of gelatin and 0.5 g of chromalum in 1000 ml of water. After drying overnight the slides were ready to receive the specimens. Early experiments showed that mounting whole cells on the slides was impractical, so it was decided to use the following technique. The growing tip of the cell was gripped with a pair of forceps while the rhizoidal portion containing the nucleus was removed. With enucleated cells, either end of the cell may be removed. By running a second pair of forceps down the stalk, the cytoplasm was squeezed out into a small drop at the cut end of the cell (Figure 4). Care must be taken not to squeeze the cell too tightly as it may break. No attempt was made to get all the cytoplasm out of the cell as it was quite impractical and was certainly unnecessary. The drop of cytoplasm was touched to the slide and was spread into a thin layer with a needle. It was found that if such drops of cytoplasm were touched to a bacterial growth medium (i.e., beef extract, yeast extract, nutrient broth, etc.), no bacterial growth occurred after a week in light or dark, at room temperature or at 37 °C. It seems safe to assume that no bacteria were being transferred with the cytoplasmic drops.

Each cytoplasm-bearing slide was treated in one of the following ways after being placed in cold 12% trichloroacetic acid (TCA) for 10 minutes and then being rinsed in distilled water for 5 minutes.

**DNase:** Some of the slides were treated with DNase (Mann Research Laboratories, Inc., 136 Liberty St., New York 6, New York, 20,000 Dornase
units/mg). Of the several combinations of time and concentration tried, a three-hour treatment with 0.3 mg of DNase/ml of water, with 0.001M MgSO₄ added, at room temperature seemed to give the best results. The slides were washed with cold 12% TCA for 5 minutes to remove the solubilized label.

**RNase**: Other slides were treated with ribonuclease (RNase) (Mann Research Labs., 44 Kunitz units/mg). Again, 0.3 mg of RNase/ml of water, for three hours, at room temperature gave best results. This was followed by a treatment with cold TCA as with the DNase slides.

**Controls**: The remaining slides were treated with TCA alone.

The slides were dried in vacuum for several hours to remove all water and were ready for coating with radioautographic emulsion.

**Radioautography.** A procedure suggested by Kopriwa and Leblond (1962) was used for the radioautography. The slides were hung by clothespins on a line in the dark room. The emulsion (NTB2 Nuclear Track Emulsion, Eastman Kodak, Rochester, New York) was heated to 40 °C in a water bath for one hour and then the slides were dipped in the emulsion in the dark and allowed to dry for one hour. The slides were then placed in slide boxes and stored in the dark in a refrigerator for one week. After this exposure period, the slides were developed in Kodak's D-17 developer, fixed, washed, and dried. The location of the silver grains was determined by examination of the slides under a microscope using oil immersion.

**Preparation of Acetabularia cells for radiochemical analysis.** For these experiments, cells 3-5 cm long were sterilized as for the radioautography. Some of the cells were enucleated by severing the rhizoid areas and the others were left intact (Figure 3). The longer cells
were easier to manipulate and fewer cells were lost during cutting. Again, at least four days were allowed for recovery of the cut cells.

Radiochemical analysis. In each of the following experiments, separate portions of 20 nucleated and 20 enucleated cells were used. The cells were placed in 5 ml of growth medium containing either C\textsuperscript{14}-thymidine or C\textsuperscript{14}-thymine (New England Nuclear Corp., Sp. Act. 45.4 mC/mM) at a concentration of 2.5 μC/ml. Incubation time in all experiments was 4 days. After this time, the cells were placed in about 100 ml of normal growth medium for one hour to remove any excess label from the cells' exterior. The contents of the enucleated cells were next transferred to one ml of cold 12% TCA in a small centrifuge tube. This was accomplished by squeezing the cytoplasm of each of the 20 cells into drops as described in the radioautography section and touching these drops to the surface of the TCA in the tube. This process was repeated for the nucleated cells. The two tubes were centrifuged at 3000Xg for 10 minutes. The supernate from each tube was removed and placed in a separate beaker to be counted. The pellet was resuspended in a one-ml portion of cold TCA and centrifuged. The process was repeated twice more with TCA and once with distilled water. Each time the supernate was pooled with the first washing. This process was found to remove all the acid soluble label from the chloroplast pellet. The pellet was next suspended in one ml of DNase (0.3 mg/ml distilled water, with 0.001M MgSO\textsubscript{4}) for three hours at room temperature. The washing and centrifuging steps were repeated and again the supernates were pooled. Again the last wash was distilled water. The pellet was next suspended in one ml of RNase (0.3 mg/ml of distilled water) for three hours at room temperature. Again the TCA washing and centrifuging steps were repeated and the supernates
pooled. Finally, the pellet was dissolved in one ml of 88% formic acid. The procedure is summarized in Figure 5. Two-tenths of a milliliter aliquots of each pooled supernate and of the pellet solution were placed on planchets, dried, and counted with a G.M. counter (Lionel 455, Lionel Electronics Labs., Brooklyn, New York, efficiency 2.5%). It was thus possible to determine the amount of acid insoluble label that was DNase soluble, RNase soluble, and insoluble.
Figure 1. Growth of the Cell

Figure 2. Preparation of the Cells for Radioautography

Figure 3. Preparation of the Cells for Radiochemistry
Remove Rhizoid Area

Slide Tweezers Down Stalk

Microscope Slide

or

1 ml Centrifuge Tube

Figure 4. Squeezing Out the Cytoplasm
Contents of 20 cells squeezed into 1 ml of cold 12% TCA

- Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernate
- Wash, 1 ml cold TCA
  - Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernate
- Wash twice more with cold TCA
  - Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernates
- Wash, 1 ml distilled water
  - Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernates
- Pooled

Suspend in 1 ml DNase, 3 hours, room temperature

- Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernate
- Wash, 1 ml cold TCA
  - Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernate
- Wash twice more with cold TCA
  - Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernates
- Wash, 1 ml distilled water
  - Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernates
  - Pooled
- Supernates

Suspend in 1 ml RNase, 3 hours, room temperature

- Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernate
- Wash, 1 ml cold TCA
  - Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernate
- Wash twice more with cold TCA
  - Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernates
- Wash, 1 ml distilled water
  - Centrifuge, 3000Xg, 10 minutes
  - Pellet
  - Supernates
- Pooled
  - Supernates

Dissolve in 1 ml 88% formic acid

Figure 5. Radiochemical Analysis
RESULTS

Radioautographic experiments. Initial radioautographic experiments were undertaken to determine conditions under which the chloroplasts of Acetabularia mediterranea would take up labeled thymidine. The earliest experiments used incubation times of 14, 26, and 52 days. All chloroplasts on all slides showed very heavy uptake of label. Background grains in areas within the cytoplasmic smears were somewhat higher than in areas of the slide not covered by the smears. This indicates that a small part of the thymidine was either utilized into DNA in cytoplasmic bodies other than chloroplasts or metabolized into some substance other than DNA. Due to the length of the incubation times, the latter case seems to be the most likely. Subsequent experiments showed that a four-day incubation period was sufficient to produce the necessary level of chloroplast label.

It was very clear that the label was being taken up into the chloroplasts. While thymidine is generally considered to be a satisfactory tracer substance for DNA synthesis, at least one investigator, Brachet (1959), obtained results which indicate, at least in Acetabularia, that the thymidine may undergo structural changes soon after it enters the cell and hence may be incorporated into some substance other than DNA. To make sure that this had not happened, it was necessary to try to remove the label from the chloroplasts with DNase.

Some difficulty was encountered in demonstrating radioautographically the removal of the label with DNase. At first, slides with cytoplasmic smears were placed in DNase solutions prior to coating with the NTB2 emulsion. Many combinations of conditions were tried without
success. Concentrations of DNase were varied from 0.1 mg/ml to 1.0 mg/ml. Distilled water and growth medium were tried as solvents for the DNase. In some cases, 0.001M MgSO\textsubscript{4} was added to the DNase solution. Slides were left in the DNase solutions from 3 to 24 hours. Temperatures were varied from 20 °C to 40 °C. None of these conditions brought about appreciable reduction in the number of grains in the emulsion over the chloroplasts.

The following conditions were found to significantly reduce the amount of label in the chloroplasts: slides bearing cytoplasmic smears were dipped in cold 12% TCA for 10 minutes, rinsed several times in distilled water, and placed in DNase (0.3 mg/ml of distilled water with 0.001M MgSO\textsubscript{4} added) for three hours at room temperature. Control experiments showed that the TCA wash removed some of the label and the DNase removed almost all the rest. Quantitative estimation of the amount of label removed by the DNase was very difficult because the chloroplasts were somewhat disrupted by the TCA treatment.

Slides bearing cytoplasmic smears of cells that had been placed in H\textsuperscript{3}-thymine (2.5 \( \mu \text{C}/\text{ml of growth medium} \)) for 4\( \frac{1}{2} \) days also showed similar removal of label following treatment with DNase as above.

Radiochemical experiments. The use of C\textsuperscript{14} labeled thymidine and thymine offered a chance to determine quantitatively the amount of label that is incorporated into DNA. The results are given in the following tables I-VI. In all these tables, the column headed "% Removed by Treatment" refers to the percent of the label removed by the TCA washes after the enzyme treatments following the initial TCA washes. Experiment #1 was actually a preliminary experiment that did not work, but it is reported here as a control. The enzyme treatments were carried out at too
high a temperature, thus inactivating the enzymes. The low number of counts obtained from the aliquots of the DNase and RNase treatments indicate that the procedure is sound and that it is indeed the enzymes that are solubilizing the label in the other experiments.

Table I. Radiochemical Analysis Experiment #1

C\textsuperscript{14}-Thymidine #1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Counts/minute 0.2 ml aliquot</th>
<th>Counts/minute Total</th>
<th>% Removed by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA Wash</td>
<td>5729</td>
<td>143,225</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>35</td>
<td>875</td>
<td>3.9</td>
</tr>
<tr>
<td>RNase</td>
<td>38</td>
<td>950</td>
<td>4.2</td>
</tr>
<tr>
<td>Pellet</td>
<td>4160</td>
<td>20,800</td>
<td>91.9</td>
</tr>
</tbody>
</table>

Total counts after initial TCA wash 22,625 100.0
Total counts incorporated 165,850
### Table IIa. Radiochemical Analysis Experiment #2

$^{3}H$-Thymidine #2.

**Enucleated Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Counts/minute 0.2 ml aliquot</th>
<th>Counts/minute Total</th>
<th>% Removed by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA Wash</td>
<td>7565</td>
<td>189,125</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>1262</td>
<td>31,550</td>
<td>60.4</td>
</tr>
<tr>
<td>RNase</td>
<td>774</td>
<td>19,350</td>
<td>37.1</td>
</tr>
<tr>
<td>Pellet</td>
<td>261</td>
<td>1,305</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Total counts after initial TCA wash 52,205
Total counts incorporated 241,330

### Table IIb.

**Nucleated Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Counts/minute 0.2 ml aliquot</th>
<th>Counts/minute Total</th>
<th>% Removed by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA Wash</td>
<td>9317</td>
<td>232,915</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>689</td>
<td>17,225</td>
<td>75.0</td>
</tr>
<tr>
<td>RNase</td>
<td>205</td>
<td>5,125</td>
<td>22.3</td>
</tr>
<tr>
<td>Pellet</td>
<td>130</td>
<td>650</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Total counts after initial TCA wash 23,000
Total counts incorporated 255,915
### Table IIIa. Radiochemical Analysis Experiment #3

**C<sup>14</sup>-Thymidine #3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Counts/minute 0.2 ml aliquot</th>
<th>Counts/minute Total</th>
<th>% Removed by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA Wash</td>
<td>5845</td>
<td>146,125</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>905</td>
<td>22,625</td>
<td>66.8</td>
</tr>
<tr>
<td>RNase</td>
<td>384</td>
<td>9,600</td>
<td>28.3</td>
</tr>
<tr>
<td>Pellet</td>
<td>340</td>
<td>1,700</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Total counts after initial TCA wash: 33,925
Total counts incorporated: 180,050

### Table IIIb.

**Nucleated Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Counts/minute 0.2 ml aliquot</th>
<th>Counts/minute Total</th>
<th>% Removed by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA Wash</td>
<td>1715</td>
<td>42,875</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>313</td>
<td>7,825</td>
<td>58.8</td>
</tr>
<tr>
<td>RNase</td>
<td>194</td>
<td>4,850</td>
<td>36.5</td>
</tr>
<tr>
<td>Pellet</td>
<td>125</td>
<td>625</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Total counts after initial TCA wash: 13,300
Total counts incorporated: 56,175
Table IVa. Radiochemical Analysis Experiment #4

$^{14}$C-Thymine #1

**Enucleated Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Counts/minute 0.2 ml aliquot</th>
<th>Counts/minute Total</th>
<th>% Removed by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA Wash</td>
<td>4096</td>
<td>102,400</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>412</td>
<td>10,300</td>
<td>46.2</td>
</tr>
<tr>
<td>RNase</td>
<td>171</td>
<td>4,275</td>
<td>19.2</td>
</tr>
<tr>
<td>Pellet</td>
<td>1544</td>
<td>7,720</td>
<td>34.6</td>
</tr>
<tr>
<td><strong>Total counts after initial TCA wash</strong></td>
<td><strong>22,295</strong></td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td><strong>Total counts incorporated</strong></td>
<td><strong>124,695</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table IVb.

**Nucleated Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Counts/minute 0.2 ml aliquot</th>
<th>Counts/minute Total</th>
<th>% Removed by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA Wash</td>
<td>4860</td>
<td>121,500</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>932</td>
<td>13,300</td>
<td>80.8</td>
</tr>
<tr>
<td>RNase</td>
<td>130</td>
<td>3,250</td>
<td>11.4</td>
</tr>
<tr>
<td>Pellet</td>
<td>453</td>
<td>2,265</td>
<td>7.8</td>
</tr>
<tr>
<td><strong>Total counts after initial TCA wash</strong></td>
<td><strong>18,815</strong></td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td><strong>Total counts incorporated</strong></td>
<td><strong>140,315</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table Va. Radiochemical Analysis Experiment #5

$^{14}$-Thymine #2

**Enucleated Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Counts/minute 0.2 ml aliquot</th>
<th>Counts/minute Total</th>
<th>% Removed by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA Wash</td>
<td>2603</td>
<td>65,075</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>322</td>
<td>8,050</td>
<td>41.1</td>
</tr>
<tr>
<td>RNase</td>
<td>185</td>
<td>4,625</td>
<td>23.1</td>
</tr>
<tr>
<td>Pellet</td>
<td>1402</td>
<td>7,010</td>
<td>35.8</td>
</tr>
</tbody>
</table>

Total counts after initial TCA wash 19,685
Total counts incorporated 84,760

Table Vb.

**Nucleated Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Counts/minute 0.2 ml aliquot</th>
<th>Counts/minute Total</th>
<th>% Removed by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA Wash</td>
<td>3252</td>
<td>81,300</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>501</td>
<td>12,575</td>
<td>42.2</td>
</tr>
<tr>
<td>RNase</td>
<td>380</td>
<td>9,500</td>
<td>32.0</td>
</tr>
<tr>
<td>Pellet</td>
<td>1536</td>
<td>7,680</td>
<td>25.8</td>
</tr>
</tbody>
</table>

Total counts after initial TCA wash 29,705
Total counts incorporated 111,005
### Table VIa. Radiochemical Analysis Experiment #6

**C\textsuperscript{14}-Thymine #3**

<table>
<thead>
<tr>
<th>Enucleated Cells</th>
<th>Counts/minute</th>
<th>Counts/minute</th>
<th>% Removed by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.2 ml aliquot</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>TCA Wash</td>
<td>3770</td>
<td>94,250</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>588</td>
<td>14,700</td>
<td>51.5</td>
</tr>
<tr>
<td>RNase</td>
<td>250</td>
<td>9,500</td>
<td>21.7</td>
</tr>
<tr>
<td>Pellet</td>
<td>1536</td>
<td>7,680</td>
<td>26.8</td>
</tr>
</tbody>
</table>

Total counts after initial TCA wash: 28,620
Total counts incorporated: 122,370

### Table VIb.

<table>
<thead>
<tr>
<th>Nucleated Cells</th>
<th>Counts/minute</th>
<th>Counts/minute</th>
<th>% Removed by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.2 ml aliquot</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>TCA Wash</td>
<td>3278</td>
<td>81,950</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>585</td>
<td>14,635</td>
<td>47.7</td>
</tr>
<tr>
<td>RNase</td>
<td>387</td>
<td>9,680</td>
<td>31.4</td>
</tr>
<tr>
<td>Pellet</td>
<td>1285</td>
<td>6,425</td>
<td>20.9</td>
</tr>
</tbody>
</table>

Total counts after initial TCA wash: 30,740
Total counts incorporated: 112,690
DISCUSSION

Discussion of the Results with Thymidine

Radioautography demonstrated the fact that chloroplasts of *Acetabularia mediterranea* incorporate labeled thymidine. Indeed, the grains in the emulsion over the chloroplasts were too numerous to count. For this reason, radioautography did not lend itself well to quantitative estimations of the amount of DNA in the chloroplast. It was also found that after DNase and TCA treatments, individual chloroplasts were no longer distinguishable. The treatments apparently broke down the chloroplast membranes and left only faint patches of green. Slides in this condition were unphotographable so no photographs could be included to illustrate the radioautographic results. This condition also added to the difficulty of making quantitative estimations of amount of label removed by DNase. It seemed that at least half of the label was being removed by DNase and most of the rest was removed by RNase.

The greater number of background grains over areas of the slide covered by cytoplasmic smears, as compared with those areas not covered by the smears, can be explained in one or both of two ways. 1.) Recent studies, reviewed by Gibor and Granik (1964), indicate that mitochondria may synthesize some DNA. 2.) The results with C\textsuperscript{14}-thymidine given in Tables II and III indicate that some of the thymidine undergoes a transformation, possibly to cytidine or uridine, and is then incorporated into RNA.

Radiochemical analysis using C\textsuperscript{14}-thymidine made it possible to determine the amounts of label in the DNA and RNA. Using the number of
counts per minute obtained from the DNase treatment and making several assumptions, one can obtain an approximation of the amount of DNA in each chloroplast. The calculation is made as follows:

\[
\frac{45.4 \text{ mC}}{\text{mM thymidine}} (\text{True sp. act. of thymidine}) \times 0.025 (\text{Counter efficiency}) =
\frac{1.13 \text{ mC}}{\text{mM thymidine}} (\text{Apparent sp. act. of thymidine})
\]

\[
\frac{20,000 \text{ c.p.m.}}{20 \text{ Cells}} = \frac{1,000 \text{ c.p.m.}}{\text{Cell}}
\]

\[
\frac{1,000 \text{ c.p.m.}}{\text{Cell}} \times \frac{1 \text{ mC}}{2 \times 10^9 \text{ c.p.m.}} \times \frac{1 \text{ mM thymidine}}{1.13 \text{ mC}} \times \frac{323 \text{ mg thymidine}}{\text{mM thymidine}} = \frac{4 \text{ mg DNA}}{1 \text{ mg thymidine}} \times \frac{1 \text{ g}}{10^3 \text{ mg}} \times \frac{1 \text{ Cell}}{4 \times 10^6} = 1.4 \times 10^{-13} \text{ g DNA/Chloroplast.}
\]

Although assumptions were made in arriving at the above figure, it is probably of the right order of magnitude. The value of the number of chloroplasts/cell was arrived at by counting aliquots of a solution containing these chloroplasts. It is doubtful if this value is off by a factor of more than 2. The value for the amount of thymidine in DNA should not be off by more than a factor of 0.1. The number of counts/minute used was an average of the number of counts/minute obtained from the enucleated and nucleated cells in both experiments. Since the number of counts varied from 31,550 to 7,825, the variability factor from the average would be on the order of 2 to 2.5 and part of this could be accounted for by a difference in the number of chloroplasts used in each experiment. This would make the variability factor for the equation 4 to 5. The value of \(1.4 \times 10^{-13}\) g of DNA per chloroplast would therefore appear to be reasonably correct within these limits.

It is interesting to compare these results with those of other investigators. With Acetabularia, Baltus and Brachet (1963) reported a
value of 0.0037-0.0059 μg of DNA from the chloroplasts of a single alga. Using the assumption that there are 4,000,000 chloroplasts per cell, this gives a value of $9.3 \times 10^{-16}$ g of DNA/chloroplast. Gibor and Izawa (1963) reported a value of $1.0 \times 10^{-16}$ g of DNA/chloroplast. Chiba and Sugahara (1957) reported values of DNA phosphorous as $3.8-6.9 \times 10^{-12}$ mg/plastid for spinach and $8.0-11.4 \times 10^{-12}$ mg/plastid for tobacco. This is converted to g of DNA/plastid by dividing by 10. Sager and Ishida (1963) reported $1.2 \times 10^{-14}$ g of DNA/chloroplast of Chlamydomonas. These and other results are summarized in Table VII below. Other investigators express their findings in other ways which are difficult to equate with these results.

**Table VII.**

**DNA Content of Chloroplasts**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Amount/Chloroplast</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach</td>
<td>$3.8-6.9 \times 10^{-13}$ g</td>
<td>Chiba &amp; Sugahara (1957)</td>
</tr>
<tr>
<td>Tobacco</td>
<td>$8.0-11.4 \times 10^{-13}$ g</td>
<td>Ibid</td>
</tr>
<tr>
<td>Chlamydomonas</td>
<td>$1.2 \times 10^{-14}$ g</td>
<td>Sager &amp; Ishida (1963)</td>
</tr>
<tr>
<td>Spinach</td>
<td>$1.5-2.5 \times 10^{-15}$ g</td>
<td>Pollard (1963)</td>
</tr>
<tr>
<td>Euglena</td>
<td>$2.3 \times 10^{-15}$ g</td>
<td>Braverman &amp; Eisenstadt (1964)</td>
</tr>
<tr>
<td>Acetabularia</td>
<td>$9.3-14.8 \times 10^{-16}$ g</td>
<td>Baltus &amp; Brachet (1963)</td>
</tr>
<tr>
<td>Acetabularia</td>
<td>$1.0 \times 10^{-16}$ g</td>
<td>Gibor &amp; Izawa (1963)</td>
</tr>
<tr>
<td>Acetabularia</td>
<td>$1.4 \times 10^{-13}$ g</td>
<td>This Thesis</td>
</tr>
</tbody>
</table>

**DNA Content of Other Cells**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Amount</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinia Virus</td>
<td>$2.0 \times 10^{-16}$ g/particle</td>
<td>Gibor &amp; Izawa (1963)</td>
</tr>
<tr>
<td><em>Aerobacter aerogenes</em></td>
<td>$2.0 \times 10^{-15}$ g/cell</td>
<td>Ibid</td>
</tr>
<tr>
<td>Sponge</td>
<td>$1.1 \times 10^{-13}$ g/cell</td>
<td>Ibid</td>
</tr>
<tr>
<td>Euglena</td>
<td>$2.5-4.3 \times 10^{-12}$ g/cell</td>
<td>Gibor &amp; Granik (1964)</td>
</tr>
<tr>
<td>Bean</td>
<td>$18 \times 10^{-12}$ g/cell</td>
<td>Ibid</td>
</tr>
<tr>
<td>Amphibian</td>
<td>$168 \times 10^{-12}$ g/cell</td>
<td>Gibor &amp; Izawa (1963)</td>
</tr>
</tbody>
</table>
Discussion of the Results with Thymine

Investigators generally agree that the free base thymine is not well utilized in DNA synthesis in either plants or animals. Brachet (1959) noted that thymine was incorporated into a "nucleic acid fraction" in Acetabularia. The results obtained in the investigation reported here confirm that Acetabularia will incorporate thymine. The radioautographic experiments show that the primary site of cytoplasmic incorporation is the chloroplast. Although the radioautography proved ineffective in quantitatively determining the amount of label that went into DNA, the C\(^{14}\)-labeled base gave the needed results. The most striking difference between the results obtained using C\(^{14}\)-thymine and those using C\(^{14}\)-thymidine is the considerably higher residual label in the pellet of the thymine. This would indicate that the thymine undergoes much more rapid and extensive metabolic changes and is incorporated into fractions other than the nucleic acids. This situation probably added to the difficulty of using radioautography quantitatively.

It can be seen in the results of the first experiment (Table IVb) that the percentage of label that was DNase removable is inordinately high when compared with the percentages in the other experiments. Since the percentages of RNase removable label and residual (pellet) label are low, it seems possible that some of the pellet may have been accidentally removed with the supernate after one of the TCA washes.

The Impact of These Results on the Theory of Chloroplast Evolution

If the chloroplast did evolve from a blue-green alga-like cell living symbiotically within a primitive protozoan, one would expect to find at least remnants of the alga's genetic material in the modern
chloroplasts. A chloroplast genetic system separate from the cell's nuclear genetic system is also necessary to explain many aspects of the chloroplast's autonomous behavior. For instance, the enucleated cells used in the first thymidine experiment had been enucleated for only a couple of weeks. The cells used in the second experiment had been enucleated for over three months. This is near the end of an enucleated cell's life span. Yet these "old" cells were still able to incorporate an appreciable amount of labeled thymidine into their chloroplasts. Since it has been known for some time that the chloroplasts increase in numbers in enucleated Acetabularia cells, one would expect incorporation of DNA precursors into these chloroplasts. However, only if the chloroplast were an autonomous organelle would one expect this incorporation to continue for so long after enucleation.

The positive evidence for the presence and synthesis of DNA in the chloroplast presented in this thesis provides important support for the theory of chloroplast evolution.

Preliminary results with radioautography showing incorporation of labeled uridine into chloroplasts and results of RNase treatments in the radiochemical experiments where a substantial amount of the label was removed by RNase, indicate the presence and synthesis of RNA in the chloroplasts of Acetabularia. Nugent (1964) has clearly demonstrated protein synthesis in Acetabularia chloroplasts. The chloroplasts of Acetabularia thus appear to contain all of the basic necessities for carrying on an autonomous existence. The next step, of course, is to attempt to grow chloroplasts in a cell-free situation.
SUMMARY

In an attempt to test the theory that the chloroplast is a potentially autonomous organelle, experiments were undertaken to locate genetic material in the chloroplasts of enucleated cells of Acetabularia mediterranea. The results of a combination of radioautographic and radiochemical techniques established the synthesis of DNA in these chloroplasts. A semi-quantitative estimation indicated that the amount of DNA per chloroplast was $1.4 \times 10^{-13}$ g. It was found that these chloroplasts would incorporate the free base thymine into DNA. Finally, these experiments indicate conclusively the presence and synthesis of RNA in these chloroplasts.


