PROTEIN SYNTHESIS BY THE CHLOROPLASTS OF ACETABULARIA MEDITERRANEAN

NOEL ATLEE NUGENT
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PROTEIN SYNTHESIS BY THE CHLOROPLASTS
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BY
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INTRODUCTION

Investigators in the field of plant biology have debated the status of the photosynthetic organelle, the chloroplast, for many years. Some have held it to be an organelle dependent upon the products of cellular metabolism for its existence while others have considered it to be independent of the cell and competent to carry on its own necessary functions within its boundaries. Current investigations have indicated, although not without ambiguity, that the chloroplast possesses its own genetic system and is capable of synthesizing the proteins pertinent to its various functions.

Previous reports on protein synthesis by chloroplasts have failed to define the purity of the chloroplast preparation with respect to contamination by cytoplasmic or nuclear protein synthetic factors (ribosomes, messenger RNA etc.). This work has also failed to demonstrate unequivocally, that protein synthesis has actually occurred. What has been reported is a binding of amino acids or other precursors in such a way that they are not removable with dilute acid. These molecules could be in a peptide linkage (protein) or they could be in lipid via a metabolic degradation and reutilization process. A recent report also indicates that amino acids may be incorporated directly into lipid material.

In all previous work chloroplasts have been isolated by breaking cells using various physical methods and separating the chloroplasts by various forms of centrifugation. Differential density and density gradient centrifugation in both aqueous and non-aqueous media have been employed. Under
these conditions denaturation and loss of protein is certain to occur.

In the experiments reported in this thesis nuclear intervention in chloroplast metabolism was completely excluded by the use of enucleated cells of *Acetabularia mediterranea* as the experimental organism. In addition, improved, gentle methods were used to remove chloroplasts from the *Acetabularia* cells and to separate chloroplasts from such cytoplasmic fractions as ribosomes and RNA.

An unequivocal demonstration of protein synthesis by chloroplasts is presented in this thesis. The impact of this finding upon present views of chloroplast status is discussed and an explanation for the autonomy of the modern chloroplast is attempted in terms of a general theory of chloroplast evolution.
LITERATURE REVIEW

Sacha (1862), Lakon (1916), Molish (1916), Meyer (1918), and Ullrich (1924) used crude staining methods and microchemical analysis to show that the majority of leaf proteins are associated with the chloroplasts. They inferred that synthesis of protein by the plastids was responsible for this accumulation. Schumacher (1929) doubted the validity of these observations because of the faulty measurements and the crude techniques used.

Granick (1938) extracted chloroplasts from several plants and determined the nitrogen content by standard kjeldahl digestion. He reported that a parenchyma cell increased its protein nitrogen ten-fold while the cell volume increased eight-fold. Some 40% of the protein was in the chloroplast fraction. This is suggestive of protein synthesis by the chloroplast. DeDecken-Gronson (1954), Mago and Jagendorf (1961), and Brawerman, et al (1962) showed similar increases in chloroplast protein under the influence of light.

Brawerman and Chargaff (1959) demonstrated that when dark-grown Euglena were placed in the light, no net protein synthesis could be detected.

Instead, a redistribution of protein occurred in favor of the developing chloroplast. To further confuse the issue Allen et al (1955) and Gibb's and Cynkin (1958), using isolated chloroplasts, showed that Cl4O2 was incorporated exclusively into glucose or starch and not into protein.

However, Sisakian and Filipovich (1955), Brachet, et al (1955), Nichiporovich (1956), Stephenson, et al (1956), and
Sisakian (1958) demonstrated incorporation of carbon-14 precursors into chloroplast proteins both in vivo and in cell free systems. It was not evident in these investigations that actual synthesis of proteins occurred in the chloroplasts.

Smillie and Fuller (1960), Smillie and Krotkov (1961), Smillie (1962), and Smillie (1963) demonstrated in greening Euglena that an increase in activity of certain enzymes, (including an alkaline fructose-1,6-diphosphatase) was associated with an increase in photosynthetic activity.

Hall et al. (1959) showed that a similar relation exists between ribulose-1,5-diphosphate carboxylase and greening of barley leaves. Brawerman and Konigsberg (1960) correlated an increase in chlorophyll content with an increase in activity of NADP⁺-glyeraldehyde-3-phosphate dehydrogenase in greening Euglena. These results are summarized by Smillie (1963).

Clark (1958), Markos (1959), and Bové and Raacke (1959) demonstrated amino acid activating enzymes in isolated chloroplasts. Lyttleton (1962), and Brawerman (1962), extracted ribosomes from chloroplasts which had been isolated from spinach and Euglena respectively. The assumption of purity of the ribosomes obtained by these methods was based on their base ratios and sedimentation coefficients which differed slightly from those of normal cytoplasmic ribosomes.

Jacobson, et al. (1963), and Murakami (1963) used cytochemical and electron microscope techniques to demonstrate RNA and "ribosome-like" structures in chloroplasts.

Heber (1962) noted that C¹⁴O₂ was incorporated into soluble chloroplast protein without any appreciable lag.
Sisakian, et al (1963), however, obtained an initial rapid incorporation of amino acids into the lipid portion of a lipo-protein complex. The amino acids appeared in protein after a short interval.

Finally Eisenstadt and Brawerman (1963), App and Jagendorf (1963), and Eisenstadt and Brawerman (1964), described a cell-free amino acid incorporating system composed of ribosomes and soluble factors derived from chloroplasts.

Many of the above mentioned papers are in direct conflict and do not conclusively prove the existence of a chloroplast protein-synthesizing system totally independent of cytoplasmic factors.
METHODS AND MATERIALS

Experimental organism. Acetabularia mediterranea was selected as the experimental organism because it can be cleanly enucleated and because of the ease with which its chloroplasts can be isolated.

This organism is a green alga (Chlorophyta) belonging to the family Dasycladaceae. It is a single cell 2-4 cm. in length when mature, composed of a "rhizoid-area" containing the nucleus, a stalk with a large vacuole and many thousands of chloroplasts, and an umbrella-like cap which eventually bears reproductive cysts. For a complete discussion of the organism see Hämmerling (1931), (1944), and Brachet (1961).

Culture. The organisms were cultured from material supplied by Dr. Konrad Keck (Johns Hopkins University, Baltimore, Maryland). The culture method is described by Keck (1964).

Culture media. The Acetabularia were grown in a medium composed of 39.9gms/liter "Seven-seas Marine Mix" (Utility Chemical Company, Paterson, New Jersey), 2ml/liter of a salt concentrate described by Keck (1964). 0.1gm/liter EDTANa₂, 0.5gm/liter tris, adjusted to pH 7.5 with HCl. All media was autoclaved 20 minutes at 15 pounds prior to using.

Removal of chloroplasts from cells. Cells selected for chloroplast isolations were 2-4 cm. in length. The nucleus of the cell was removed by amputation of the rhizoid area. The contents of the stalk were expelled into a small container of culture medium diluted 1:1 with distilled water by squeezing the cell with small forceps. The contents of about 20 cells were expressed into 2 ml. of medium for each experiment.
All operations of this type were performed in a sterile transfer hood. The sterility of protoplasts prepared by this technique was tested by placing drops of protoplasm on plates of agar containing a good bacterial growth medium, i.e. nutrient broth, yeast extract, beef extract, etc. The plates were then divided into two groups and incubated at either room temperature or 37°C. Incubation was allowed to continue for up to a week. These tests indicate that no bacteria were being transferred with the protoplasm of the alga.

In many experiments freshly removed protoplasm with no further purification was used.

**Determination of chlorophyll in bottom phase.** The total chlorophyll present in the bottom phase of the purification system was determined in terms of the optical density of an ether extract as measured at a wavelength of 660 m\(\mu\) in a Beckman DU spectrophotometer.

**Determination of RNA in the bottom phase.** The total RNA of the bottom phase was determined in terms of the optical density of a ribonucleotide extract following hydrolysis of the bottom phase in 0.1N KOH at 40°C for 2 hours. The optical density was measured at a wavelength of 260 m\(\mu\) in a Beckman DU spectrophotometer.

**Radioautography.** Tritium labeled leucine (Schwarz Bioresarch Incorporated, Orangeberg, New York) with a specific activity of 0.5 curies per millimole was introduced into the chloroplast growth medium at a concentration of 10\(\mu\)C/ml. The chloroplasts were incubated at room temperature in constant light from an incandescent source. Following an incubation period of 12
hours an equal volume of cold 12% trichloroacetic acid (TCA) was added and the suspension centrifuged at 3000xg for 10 minutes at 0°C. After three additional TCA extractions the pellet was smeared on a microscope slide and coated with Kodak NTB2 emulsion in accordance with the method of Kopriwa and LeBlond (1962). The slides were stored in the dark in a refrigerator for seven days and then developed. The number of grains over the chloroplasts were counted and recorded as grains per 1000 square microns. Background radiation was determined by counting grains on a coated portion of the slide which was free of any pellet material (unexposed to internal radiation). Non-chloroplast incorporation was determined by counting grains over smeared areas devoid of chloroplasts. This process is depicted diagrammatically in figure 2.

Radiochemical assay. C\textsuperscript{14}-labeled leucine (New England Nuclear Corporation, Boston, Massachusetts) with a specific activity of 240 millicuries/millimole was introduced into the chloroplast medium at a concentration of 5\textmu curies/ml. Incubation was carried out for periods of time and at temperatures specified for each experiment in the Results section. Extraction with TCA was performed as described in the section on radioautography. The TCA extracts were pooled and a small aliquot was counted (Lionel 455 scalar-ratemeter, Lionel Electronics Laboratory Incorporated, Brooklyn, New York) to obtain the total count extracted. At this point a variety of lipid solvents, specified with each experiment in the Results section, were used. Extraction was performed twice and the supernatants were combined. The supernatant was counted in small aliquots and
the results were pooled into a total count figure. The precipitate from the TCA wash was hydrolyzed 12 hours at 40° C with either trypsin or pancreatin powder followed by erepsin (Mann Research Laboratories Incorporated, New York, New York) as described by Block (1960). Initially trypsin was employed, but it was found that pancreatin was identical in its action on the incorporated leucine. TCA extractions were performed as previously described in the section on radioautography and again aliquots of the supernatant were counted. The precipitate was then suspended in 0.5 ml. of 88% formic acid and dried on a planchet to be counted.

One set of experiments followed the above form except that the lipid extraction was omitted. An additional experiment using chloroplasts which had been pretreated with RNase was performed.

The biochemical procedures used in this assay are outlined in figure 3.

The final experiments were performed on chloroplasts which had been purified. In all counting procedures the effect of self absorption was determined by placing 1 ml. of a given solution on a planchet in 0.10ml. increments. The planchet was dried and counted between increments. In most cases the error due to self absorption reached 1% after 0.3 to 0.5 ml. had been added. Calculations have shown that a 20 mg. pellet (which was the largest used) would cause a maximum error of about 3%.

Chloroplast purification. To purify the chloroplasts with respect to cytoplasmic factors including ribosomes, a biphasic system described by Albertsson (1960) was used. It was composed
of methyl cellulose (MC4000, Fisher Scientific Company, Boston, Massachusetts) and dextran (D68, Pharmacia, Uppsala, Sweden), and is identical to the Al system described by Albertsson except that half strength *Acetabularia* culture medium was used instead of the salts recommended by him. The purification scheme is illustrated in figure 1. The final partition step was performed from one to ten times depending upon the requirements of the particular experiment. For maximum purity, extractions were performed until protein could no longer be detected in the upper phase by the method of Lowry *et al* (1951) and the RNA level of the lower phase was constant by U.V. absorption.

Most of the protein and cytoplasmic RNA are diluted out with five extractions. Since an additional five extractions had little further effect on amino acid incorporation by the chloroplasts, ten extractions were routinely performed in later experiments.

The only cytoplasmic contaminants which were not removed from the chloroplast preparation by this method were the mitochondria. These organelles were assumed to be rapidly inactivated in the autotrophic medium since no energy source was available to them. In order to remove mitochondria the last step of the purification scheme (figure 1.) was performed.
Chloroplasts are expelled into culture medium

Add equal volume of mixture Al of Albertsson; mix by inverting several times.

Centrifuge at 8000xg, 45 minutes

Top phase; discard. Contains cytoplasmic ribosomes and soluble components.

Bottom phase; contains chloroplasts, mitochondria, and some remaining cytoplasm.

Repeat extractions until RNA of the bottom phase reaches a constant level; 10 extractions insure complete separation.

The final bottom phase is still contaminated with mitochondria which can be removed by layering the suspension on a 1% solution of methyl cellulose and centrifuging at 300xg for one minute. The chloroplasts sediment rapidly while the mitochondria descend quite slowly.

Figure 1.
**Enucleation.**

**Nuclear region.**

**Removal of protoplasm.**

**Drop of protoplasm.**

**Vessel of culture medium plus 10µC/ml. H²⁻-leucine.**

**Incubate 12 hrs. at 20°C in constant light.**

**Add one volume 12% TCA at 0°C.**

**Centrifuge at 3000xg, 10 minutes at 0°C.**

**Discard supernatents.**

**Repeat TCA extractions three times.**

**Final precipitate smeared on slide and coated with NTB2 emulsion.**

Figure 2.
Chloroplasts
plus C14-leucine

Incubation

TCA extraction

Supernatant was assayed for activity.

Precipitate extracted with either DOC, ether-acetic acid, or acetone-acetic acid.

Supernatant was assayed for activity.

The precipitate was hydrolyzed with protease enzymes and then extracted with the TCA.

Precipitate and supernatant was assayed for activity.

Figure 3.
RESULTS

Radioautographic experiments with H\textsuperscript{3}-leucine. The results of these experiments are summarized in table I. The contents of twenty enucleated cells were used in this experiment.

Table I

<table>
<thead>
<tr>
<th>area</th>
<th>grains/1000 \textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>background</td>
<td>8.1±0.8</td>
</tr>
<tr>
<td>nonchloroplast</td>
<td>74±6</td>
</tr>
<tr>
<td>chloroplast</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

These data show extensive incorporation by the chloroplasts and a small amount of cytoplasmic incorporation. The graining over the chloroplasts was too dense to be counted and was designated as being greater than 1000 grains/1000 \textsuperscript{2}.

Radiochemical experiments.

1. With Freshly Expelled Protoplasts

The data on these experiments are contained in table II. Figures 4 through 10 illustrate the results graphically.

Table IIa shows the radioactivity (in counts per minute) which remained in the centrifuged pellet after the extractions indicated. The experiments were run at 20\textdegree C for various time periods. Experiment number 5 was the same as experiment number 3 except that the chloroplasts were incubated at 20\textdegree C for two hours in RNase (.3mg/ml), prior to incorporation.

Table IIb shows the same experiment in terms of the percent of the total incorporation which was found in the various fractions.
Table IIa

ACTIVITY REMAINING AFTER INDICATED OPERATION

<table>
<thead>
<tr>
<th>expt. #</th>
<th>operation</th>
<th>times of incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1.</td>
<td>trichloracetic acid (TCA) extraction</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>protease hydrolysis and TCA extraction</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>TCA extraction</td>
<td>526</td>
</tr>
<tr>
<td></td>
<td>extraction by acetone-acetic acid</td>
<td>298</td>
</tr>
<tr>
<td></td>
<td>protease hydrolysis and TCA extraction</td>
<td>32</td>
</tr>
<tr>
<td>3.</td>
<td>TCA extraction</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ether-acetic acid extraction</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>protease hydrolysis and TCA extraction</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>TCA extraction</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>deoxycholate (DOC) extraction</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>protease hydrolysis and TCA extraction</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>RNase pretreatment TCA extraction</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ether-acetic acid extraction</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>protease hydrolysis and TCA extraction</td>
<td>-</td>
</tr>
</tbody>
</table>

Table IIb

% OF TOTAL ACTIVITY IN MAJOR FRACTIONS

<table>
<thead>
<tr>
<th>expt. #</th>
<th>fraction</th>
<th>times of incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1.</td>
<td>protease soluble</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>residual</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>acetone-acetic acid soluble</td>
<td>43.4</td>
</tr>
<tr>
<td></td>
<td>protease soluble</td>
<td>50.5</td>
</tr>
<tr>
<td></td>
<td>residual</td>
<td>6.1</td>
</tr>
<tr>
<td>3.</td>
<td>ether-acetic acid soluble</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>protease soluble</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>residual</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>DOC soluble</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>protease soluble</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>residual</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>RNase pretreatment ether-acetic acid soluble</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>protease soluble</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>residual</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4. This graph shows the data of experiment 2 of table IIa, plotting the activity in counts per minute against the time in minutes.

A. Activity after TCA extraction.
B. Activity after acetone-acetic acid (pH 3.0) extraction.
C. Activity after pancreatin and erepsin digestion.

These operations were successive steps in an experimental sequence.
Figure 5. This graph shows the data of experiment 2 of table IIb, plotting the percent activity against the time of incubation in minutes.

A. Percent incorporation into acetone-acetic acid (pH 3.0) soluble fraction.

B. Percent incorporation into protease soluble fraction.

C. Percent incorporation remaining after all operations.
Figure 6. This graph shows the data of experiment 3 of table IIa, plotting the activity in counts per minute against the time in minutes.

A. Activity after TCA extraction.
B. Activity after ether-acetic acid (pH 3.0) extraction.
C. Activity after pancreatin and erepsin digestion.
Figure 7. This graph shows the data of experiment 3 of table IIb, plotting the percent activity against the time of incorporation in minutes.

A. Percent incorporation into ether-acetic acid (pH3.0) soluble fraction.

B. Percent incorporation into protease soluble fraction.

C. Percent incorporation remaining after all operations.
Figure 8. This graph shows the data of experiment 4 of table IIa, plotting the activity in counts per minute against the time in minutes.

A. Activity after TCA extraction.

B. Activity after 0.4% DOC extraction.

C. Activity after pancreatin and erepsin digestion.
Figure 9. This graph shows the data of experiment 4 of table IIb, plotting the percent activity against the time of incubation in minutes.

A. Percent incorporation into the 0.4% DOC soluble fraction.
B. Percent incorporation into protease soluble fraction.
C. Percent incorporation remaining after all operations.
Figure 10. This bar graph shows the data of table IIb under the 30 minute time period. The bars show a comparison of all five experiments.
2. With Chloroplasts Purified in the Biphasic Systems of Albertsson

The results of these experiments are contained in table III and figures 11 and 12. Table III shows the optical density (O.D.) of the chlorophyll extract (660 m\(\mu\)) and the optical density of the ribonucleotide extract (260 m\(\mu\)) obtained from the lower phase (see figure 1.). It also shows the results of a 90 minute incorporation test with the chloroplasts contained in the lower phase.

Table III

<table>
<thead>
<tr>
<th>expt.</th>
<th>number of extractions</th>
<th>O.D. 660 m(\mu)</th>
<th>O.D. 260 m(\mu)</th>
<th>CPM 90 min. 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0</td>
<td>0.284</td>
<td>0.962</td>
<td>8463</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.280</td>
<td>0.566</td>
<td>8194</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.285</td>
<td>0.539</td>
<td>8068</td>
</tr>
<tr>
<td>2.</td>
<td>0</td>
<td>0.248</td>
<td>0.973</td>
<td>7963</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.255</td>
<td>0.778</td>
<td>7782</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.232</td>
<td>0.608</td>
<td>7750</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.246</td>
<td>0.571</td>
<td>7891</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.228</td>
<td>0.551</td>
<td>7755</td>
</tr>
</tbody>
</table>
Figure 11. This graph shows the data from table III which concerns the first experiment involving 0, 5, and 10 extraction cycles. Curve A is the optical density (O.D.) 660mμ; curve B is the O.D. 260mμ; curve C is the incorporation of C\textsuperscript{14}-leucine in counts per minute (CPM).
Figure 12. This graph shows the data from table III which concerns the second experiment involving 0, 2, 5, 7, and 10 extraction cycles. Otherwise this graph is the same as figure 11.
DISCUSSION

1. Discussion of results

The results of the radioautographic experiments indicate that a very large amount of label goes into the chloroplasts. A small amount of label was present in the cytoplasm and was probably due to a low level incorporation by the cytoplasmic ribosomes. The reasoning here was that the medium in which this reaction was run was not ideal for free ribosomal incorporation. The concentration of divalent cations was sufficient to cause aggregation of ribosomes. Aggregated ribosomes can incorporate amino acids but only to a small degree.

The experiments illustrated in table II and figure 4 through 10 show several interesting points. The over-all incorporation increases in a nearly linear fashion over the duration of the experiments. However, while the percentage of the activity which was protease-removable was in the vicinity of 83% (figure 10 experiment 1) for a reaction time of 30 minutes, a significant portion of this label was extractable with various acidified lipid solvents (figure 10 experiments 2, 3, and 4). The label removed by acidified lipid solvents appears to be incorporated into a phospho-lipid complex since no label was removed when lipid solvents such as ether were used in the absence of acid.

It would further appear that a large portion of this phospho-lipid was actually a phospho-lipo-protein since much of the label on the phospho-lipid complex was protease removable.
The finding that the total protease removable plus acidified lipid-solvent removable activities was between 90 and 98% of the total activity indicates that between 7 and 15% of the total activity is associated directly with the phospho-lipid portion. It would also appear that the percent incorporation of label increases in protein and decreases in phospho-lipo-protein as incubation times increase. To further complicate the picture, treatment of the chloroplasts with RNase before incorporation increases the activity of the ether-acetic acid fraction to 93% of the total activity. This latter point could explain the results obtained by Eisenstadt and Brawerman (1963), (1964), which showed no inhibition of incorporation of amino acids by chloroplasts pretreated with RNase. Under these conditions most of the activity may go directly in phospho-lipo-protein (or phospho-lipo-amino acid) with very little activity in free protein.

These results point to the possible existence of three separate mechanisms of amino acid incorporation in the chloroplast. Two major pathways lead to protein and phospho-lipo-protein while a minor pathway leads to phospho-lipid. The evidence presented here cannot rule out a precursor-product relationship between these systems. It is interesting to speculate that this relationship might involve a pathway such as phospho-lipid to phospho-lipo-protein to protein. Much more investigation is needed to reveal the true relationship.

Experiments exhibited in table III and figure 11 and 12 demonstrate that the biphasic system of Albertson is effective in removing cytoplasmic RNA without significant loss
of the ability of chloroplasts to incorporate amino acids. With this method the chloroplasts are partitioned in favor of the bottom phase and sediment under the conditions employed. The sediment is easily resuspended as only gentle swirling of the solution is required. The separation of cytoplasmic ribosomes from the chloroplasts can be accomplished in two extractions if 90% of the bottom phase is removed along with the top phase.

This method removes all cytoplasmic structures smaller than 0.1μ. If contamination by mitochondria is of concern they can be removed by centrifuging the final chloroplast suspension obtained from the Albertsson method in a 1% solution of methyl cellulose. The chloroplasts sediment slowly as a band while the mitochondria remain in the top centimeter of the tube. The methyl cellulose-dextran system of Albertsson thus would appear to be ideal for chloroplast isolations and could probably be used to advantage in mitochondrial studies.

2. Impact of these Results on the Status of the Chloroplast

With the proof afforded here, that the chloroplasts of Acetabularia mediterranea can and do synthesize protein in the absence of all nuclear or cytoplasmic control, it can be established that the chloroplast is independent of the cell in this regard. The chloroplast in this respect deserves the rank of organism. Further support for this idea comes from recent work by Gibor and Izawa (1963) which indicates that the chloroplasts of Acetabularia contain a DNA synthetic system. Thus the chloroplasts of this alga contain a DNA-RNA-protein
system which is indicative of complete autonomy. Further work is needed to determine if all factors necessary for maintenance and growth of the chloroplast are independent of cellular control. Ultimately only the successful culture of chloroplasts in a defined cell-free medium will prove this to be true.

Since the evidence obtained in conjunction with this thesis and with other recent work would indicate that a chloroplast can be considered to be at the level of an organism, a working hypothesis of chloroplast origin in these terms should be useful.

3. A Theory of Chloroplast Origin

A. The Theory

Chloroplasts at one time were cells which resembled primitive blue-green algae (Cyanophyta). These primitive cells contained chlorophyll a, carotenoids, and probably phycocyanin as their major pigments. They were small (1-5μ) and were themselves only one step removed from the green photosynthetic bacteria. Certain primitive protozoans, perhaps with a phagocytic feeding habit, utilized these primitive blue-greens as a major source of food. Certain species of algae were resistant to the digestive enzymes of the protozoan and remained alive and functioning in the protozoan cell. Eventually a true symbiotic relationship became established. The algae obtained raw materials from the protozoan and the protozoan in turn received synthetic products from the algae.

As the alga continued to evolve it gained the ability to synthesize chlorophyll b and lost the ability to synthesize phycocyanin. It also evolved the ability to synthesize starch
which became the dominant form of energy storage.

In the meantime the protozoan cell was also evolving. It evolved a flagellum and eye spot (if these were not already present when the association began) and also a more rigid membrane typical of many present protozoans. It lost its phagocytic ability and became dependent upon the "chloroplast" (or symbiotic algae) for its existence.

The advantage of the evolving symbiosis are obvious. The algae was now in an environment the constancy of which was maintained and protected by the activities of the protozoan. The protozoan with its newly-acquired photosynthetic system, could exchange a life of predation for a simpler searching for minerals and light. This new, complex organism would be capable of invading ecological niches previously closed to both of the symbionts.

The idea of chloroplasts as endocellular symbionts is not new (Mereschkowsky, 1905) but a detailed theory has not been presented previously.

B. The Evidence for This Theory

The evidence for this theory (as is the case with all evolutionary theories) is one of circumstantial nature. It is, however, strong enough to make a reasonably good case.

characteristic photosynthetic pigments, chlorophyll a and β-carotene. Both also have a strikingly similar ultrastructure; in coccoid blue-greens and in green algal chloroplasts, for example, a peripheral lamellar system contains the photosynthetic apparatus while a clear central region contains the genetic material. Finally, both reproduce by the primitive process of binary fission.

It seems improbable that these similarities are coincidental. Rather they indicate a close evolutionary relationship and lend support to the theory being discussed here.

Even more convincing support for the theory is the existence today of several types of symbiotic associations involving blue-greens and protozoans. The most significant of these with regard to the theory is the case of *Cyanophora paradoxa* Pascher (1929), Hall and Claus (1963). This small biflagellated protozoan characteristically contains two blue-green algal cells of the *Synechococcus* type. Electron microscopy of the algal symbionts reveals a concentric peripheral lamellar system and the absence of a typical blue-green cell wall and cyanophycin granules. That these algae are blue-greens rather than greens is indicated by the finding that they lack the chlorophyll b typical of the greens. It is most interesting that whereas each of the symbionts separately is unable to synthesize starch, together they synthesize starch as the major storage compound. It would appear that a linkage of the metabolisms of both organisms activates a pathway which leads to the synthesis of starch.

An example of a more advanced transitional form is the
extraordinary alga *Cyanidium caldarium*, which resembles *Chlorella* in its morphology and several features of its physiology, but which contains only chlorophyll a, and in addition forms the type of phycocyanin typical of blue-green algae (Hirose, 1950, Allen, 1952, 1954, 1959). This organism must be studied more completely if its true evolutionary position is to be determined.

It may be of some significance that many green algae, for example *Acetabularia*, produce a gamete which is biflagellated and which in other respects also resembles *Cyanophora*. Could it be mere coincidence that an advanced green alga possesses all the genes necessary to produce a biflagellate cell similar to protozoan cells? Or does this rather represent an ancient ancestral ability which has been retained through the phylogeny of these algae?

C. Possible Objections to the Theory

It is possible to question the theory by emphasizing the differences between chloroplasts and blue-green algae. For example, how is it possible to explain the presence of chlorophyll b and *α*-carotene in chloroplasts when these pigments are lacking in blue-green algae? Or, again, why do chloroplasts lack the phycocyanin which is present in blue-greens?

Answers to these questions are possible within the framework of evolutionary theory. Normal processes of mutation for example could result in the evolution of chlorophyll b from chlorophyll a whereas selection could account for the preservation of the mutation in the new environment of the
symbiotic association. The reverse mutation can be seen in mutant G-44 of Chlorella pyrenoidosa (Allen et al. 1960). In this mutant the capacity to synthesize chlorophyll b has been lost. Similarly, mutation and selection can logically be invoked to explain other biochemical differences between blue-green algae and chloroplasts.

An explanation must be found for the gross differences in size and structure which exist between the blue-green algae and the chloroplasts of certain green algae. It is difficult, for example, to envisage the mechanism which produced the large spiral chloroplast of organisms such as Spirogyra. This could conceivably have come about via a coalition of several chloroplasts into a linear array and their subsequent fusion. Recent work with Nitella (Green, 1964) indicates a tendency of chloroplasts to remain attached after division. The same work also shows the ability of chloroplasts to orient themselves with respect to stress in the cell wall. If a spiral stress is present in Spirogyra walls this could account for the spiral shape of the chloroplast.

D. Areas for Future Research

It is evident from the foregoing discussion that the key to the theory may be found in Cyanophora paradoxa. It should be possible to utilize a strain free of chromatophores to analyze the biochemical makeup of the protozoan and to compare these findings with those secured from several green algae similarly free of chromatophores. Then the blue-green component of Cyanophora could be compared in a similar manner
to other *Synechococcus* species and to chloroplasts of green algae.

Another approach would be to study chloroplasts free from any nuclear or cytoplasmic factors as was attempted in this thesis, or to grow chloroplasts in a cell free medium. The latter has not yet been reported, but should not be far in the future.

It would also be of interest to see what kinds of mutations one could produce in *Cyanophora* with the aid of a variety of mutagens.
SUMMARY

It has been demonstrated that chloroplasts of *Acetabularia mediterranea* incorporate amino acids into three fractions:

1. A protease soluble fraction.
2. Phospho-lipo-protein complexes, probably in the protein portion.
3. A probable phospho-lipid complex.

The incorporation into phospho-lipid and phospho-lipo-protein is considerably higher on a percent basis after short incubation intervals than after longer intervals.

A system which effectively separates chloroplasts from other cytoplasmic constituents while having very little adverse effect upon the chloroplasts is presented.

Finally, the status of the chloroplast is discussed and a theory as to its origin is presented.
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