BIOSYNTHESIS IN ISOLATED *ACETABULARIA* CHLOROPLASTS

I. Protein Amino Acids

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ABSTRACT

The ability of chloroplasts isolated from *Acetabularia mediterranea* to synthesize the protein amino acids has been investigated. When this chloroplast isolate was presented with $^{14}CO_2$ for periods of 6–8 hr, tracer was found in essentially all amino acid species of their hydrolyzed protein Phenylalanine labeling was not detected, probably due to technical problems, and hydroxyproline labeling was not tested for The incorporation of $^{14}CO_2$ into the amino acids is driven by light and, as indicated by the amount of radioactivity lost during ninhydrin decarboxylation on the chromatograms, the amino acids appear to be uniformly labeled. The amino acid labeling pattern of the isolate is similar to that found in plastids labeled with $^{14}CO_2$ in vivo. The chloroplast isolate did not utilize detectable amounts of externally supplied amino acids in light or, with added adenosine triphosphate (ATP), in darkness. It is concluded that these chloroplasts are a tight cytoplasmic compartment that is independent in supplying the amino acids used for its own protein synthesis. These results are discussed in terms of the role of contaminants in the observed synthesis, the "normalcy" of *Acetabularia* chloroplasts, the synthetic pathways for amino acids in plastids, and the implications of these observations for cell compartmentation and chloroplast autonomy.

INTRODUCTION

Chloroplasts are thought to be semiautonomous cell organelles, perhaps derived from ancestral intracellular symbionts (see references 25 and 33 for reviews) Many chloroplasts including those of the giant unicellular alga, *Acetabularia*, have sufficient quantities of DNA to warrant the suggestion that they might be able to code for many of their own structural and enzymatic proteins (19, 49, 50). The ability of these (2, 11, 18, 40) and other chloroplasts (4, 28, 42) to synthesize such proteins in vitro is widely recognized, and it is clear that chloroplasts possess their own protein-synthesizing system (17, 45). On the other hand, chloroplasts do not carry the genetic information for all of their important constituent enzymes (25, 26, 34). Some of these nuclear-coded proteins may nevertheless be synthesized within the chloroplasts (27, 44). The presumed origin of chloroplasts, their genetic capabilities, and their high biosynthetic activity suggests that they represent a cell compartment with a protein synthetic ability that is in many respects independent of the rest of the cell. It seems reasonable to suppose that chloroplast integrity and function depend on the synthesis of some protein constituents *in situ*. If this is the case, a shortage of one or more amino acids could prevent the chloroplast from carrying out these syntheses, resulting in subsequent deleterious effects to the cell from photosynthetic inadequacy. Examples of such deleterious effects have been pro-

vided by Walles (48), using mutant barley seedlings. Nuclear mutations affecting several amino acid pathways prevent maturation of the proplastids and thus the greening and growth of the plants. If the mutant seedlings are supplied with the appropriate amino acid until the chloroplasts mature, no further supplementation may be required. This shows that the mature chloroplasts can supply amino acids which are provided by nuclear-controlled pathways for proplastids and probably for the rest of the cell. Thus, there appear to be redundant pathways leading to these amino acids, one in chloroplasts and another in a different cytoplasmic compartment. Such redundancy would seem more reasonable the more independent the chloroplasts are in providing the amino acids and other requirements to support their own protein synthesis. Free pools of several amino acids (glycine, serine, alanine, and aspartic and glutamic acids) appear as early products of photosynthesis in most chloroplasts (8, 21, 23, 41), but the numerous enzymes of the pathways for the rest of the amino acids are not usually considered chloroplast constituents.

The chloroplasts of *Acetabularia* are known to multiply in the absence of a cell nucleus (15, 36), and their biosynthetic capacity under these conditions remains quite high for several weeks (10, 11, 13, 37) Chloroplasts have been isolated from *Acetabularia* in a highly intact condition. Their in vitro photosynthetic activities are normal for many hours (7, 8, 39, 40). This isolate should provide an excellent material for studying organelle biosynthetic capacity. The performance of these chloroplasts in enucleate cells suggests that they might be capable of extensive synthesis of structural and enzymatic proteins in vitro. However, any amino acid requirements would severely limit in vitro performance unless these requirements were determined and the amino acids provided in the incubation medium. Thus, our study of biosynthesis in isolated chloroplasts begins with the metabolism of the protein amino acids.

MATERIALS AND METHODS

Chloroplast Isolation and Incubation

The cells used in this study were *Acetabularia mediterranea* which had been maintained in continuous laboratory culture by methods previously described (38). Most of these cultures were not axenic; however there were no contaminating photosynthetic species and the bacterial population was low, consisting mostly of *Pseudomonas* spp. The chloroplast isolation procedure shown in Fig. 1 was modified from that previously described (7, 39). The step gradients were made in sterile glassware, using membrane filter sterilized media. The bacterial contamination of the final pellet usually was between one bacterium per 10^6–10^8 chloroplasts by plate count, and most incubations were carried out with fewer than 100 bacteria in the starting material.

Nucleopore filters (General Electric Co., Pleasanton, Calif.) with a pore diameter of 5 μ were used to shear cytoplasmic droplets and filter out debris. Fig. 2 shows the appearance of the initial homogenate and the suspension obtained after Nucleopore filtration. It is apparent that filter shearing was very effective in eliminating cytoplasmic contamination. The isolation and incubation media (Table I) were similar to those previously described (7). Added bovine serum albumin (BSA)[1] and NO_3^- and $SO_4^=$ salts in the A medium led to better carbon fixation and chlorophyll recovery during long term incubations. An increased buffer concentration was necessary to handle the pH change due to HCO_3^- utilization. No deleterious effects of even 50 mM n-tris(hydroxymethyl)methyl-2-amino-ethane sulfonic acid (TES) have been noticed in short term incubations (16).

After considerable experimentation in the earlier incubations, the following incubation conditions were selected as standard: Chlorophyll concentration, 3–4 μg/ml; temperature, 24°C; light intensity, 400 ft-c; and no more agitation than necessary to keep the chloroplasts in suspension. Initial HCO_3^- concentrations ranged from 1 to 2 mM. The carbon supply becomes rate-limiting below 0.1 M HCO_3^- (39). Linear carbon fixation has been observed for as long as 12 hr in continuous light and at rates from 20 to 40 μmoles/hr per mg chlorophyll (Chl). Most incubations were continued for 6–8 hr; however, occasionally, on the assumption that different parts of the daily cycle are characterized by different metabolic patterns, approximately 24 hr incubations with a dark period on the normal schedule were used.

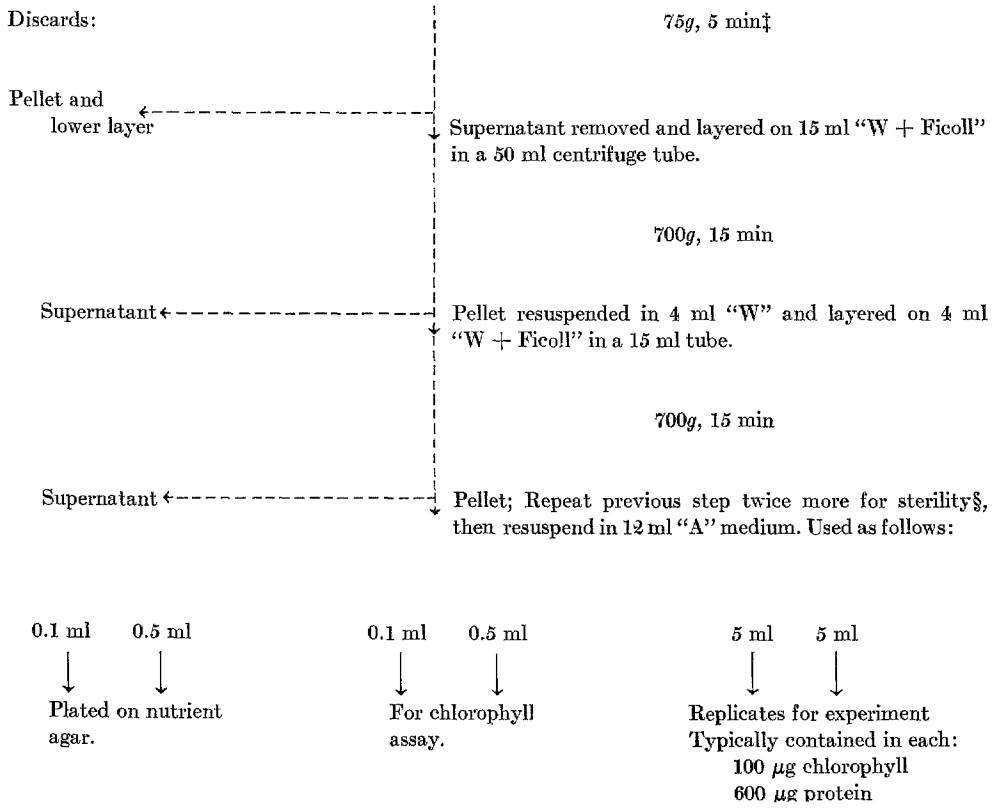
Radioisotopes

Isotopic labeling was carried out with $Na_2{}^{14}CO_3$ or $NaH^{14}CO_3$ (50–57 mCi/mmole). Unlabeled carrier $KHCO_3$ was added to bring the final concentration to 1–2 mM HCO_3^- and the specific activity to 2–25 mCi/mmole. When comparisons of incorporation were to be made, the different samples were adjusted to identical pH and sealed inside a large

[1] *Abbreviations used:* ATP, adenosine triphosphate; BSA, bovine serum albumin; Chl, chlorophyll; DTT, dithiothreitol; EDTA, disodium ethylenediaminetetraacetate; TES, n-tris (hydroxymethyl) methyl-2-amino-ethane sulfonic acid.

FIGURE 1 Chloroplast Isolation

Cells were scissor minced in "H" medium (1.5 g per 3 ml*). The slurry was introduced into a filter funnel, tamped gently, and rinsed through 173 mesh bolting cloth with 8 ml of "W" medium This crude suspension was forced through a 5 μ membrane filter attached to a hypodermic syringe. After filtering, it was layered on 15 ml of "W + Ficoll" in a 50 ml centrifuge tube. All operations were carried out on ice.



* The quantities represent a typical experiment but also maximum loading for one centrifuge tube in each step.

‡ This step removes remaining aggregates and starch-rich chloroplasts.

§ These steps separate chloroplasts from lighter components. When carried out with sterile glassware and membrane sterilized media they can be 100% effective in eliminating bacteria also.

container with a third vessel containing an excess of labeled and carrier HCO_3^- to insure that the availability and specific activity of $^{14}CO_2$ was identical for all samples during the course of the incubation. Uniformly labeled ^{14}C-amino acids were obtained from New England Nuclear Corp. (Boston, Mass.), and the artificial "algal hydrolysate" was obtained from International Chemical and Nuclear Corporation (Burbank, Calif.). The L-isomers of the amino acids were used and their concentrations and radioactivities are noted in Table VI. The identity and purity of these preparations were checked by chromatography at the time of their use.

Chlorophyll Assay

Chlorophyll determinations were made on 80% acetone extracts of chloroplast pellets by using the formula of Arnon (1). An extensive study of the pigments in these plastids has been carried out in our laboratory (30). There was no problem in total chlorophyll extraction from small pellets of isolated chloroplasts, and brief centrifugation resulted in a light scatter reading (700 nm) of 0–0.02 A units. This reading was subtracted from the chlorophyll peaks (3) There was little tendency of the extracted chlorophyll to break down and discolor as is the case

TABLE I

Composition of Media

	Mannitol	EDTA	BSA	TES	DTT	pH (with KOH)
	M	*M*	%	*M*	*M*	
H (homogenizing)	0.6	10^{-3}	0.1	0.1	10^{-3}	8.0
W (washing)	0.6	10^{-3}	0.1	5×10^{-3}	10^{-3}	7.8
A (assay)	0.6	—	0.1	2×10^{-2}	—	7.8

"A" also contains: KCl, 10^{-2} M, $MgCl_2$, 5×10^{-3} M; KH_2PO_4, 5×10^{-4} M, $MgSO_4$, 10^{-4} M; $NaNO_3$, 10^{-4} M; $KHCO_3$, $1 - 2 \times 10^{-3}$ M.
"W + Ficoll" is made by adding 2.15 g of Ficoll (Pharmacia, Uppsala, Sweden) to 100 ml of "W".

with whole cell extracts. The analysis required no more than 5 μg of total chlorophyll in a semimicro cuvette. All rates or comparisons were expressed per μg Chl.

Termination Procedure

The termination procedure is presented in Fig. 3. The low speed sedimentation used does not recover the fragments of chloroplasts lysed during the incubation. Higher speed sedimentation damages the chloroplasts and releases radioactive compounds to the supernatant. A terminal chlorophyll determination was performed on the 80% acetone extract of the whole pellet to estimate recovery. This was rarely less than 90%.

Samples of the whole suspension (A), the supernatant (B), and the 80% acetone extract (C) were put on planchets with several drops of 0.05 N HCl to drive off the $H^{14}CO_3^-$. These were dried and counted with a Nuclear-Chicago (Des Plaines, Ill.) gas flow counter with a "Micromil" window. From these counts estimates were made of total fixation (A), soluble (C), and insoluble [A − (B + C)] products and products released to the incubation medium (B). These figures are used to monitor the performance of the isolate.

The pellet was washed several times to complete the removal of water-soluble compounds and lipids (including a wash in cold 5% perchloric acid with an excess of unlabeled amino acids when ^{14}C-amino acids were used). The residue was hydrolyzed in 100°C, 6 N HCl overnight in a nitrogen atmosphere at reduced pressure. The HCl was driven off with a stream of nitrogen at 90°C, and the crude hydrolysate was redissolved in water, usually 1 μl per original μg Chl. It has been found (Levin and Shephard, unpublished) that 1 μg Chl represents approximately 6 μg plastid protein. On this basis, the hydrolysis and subsequent analysis was carried out on 200–900 μg of protein.

Chromatography

Samples of the hydrolyzed material (1–5 μl) were analyzed by thin-layer chromatography (20 × 20 cm plastic sheets with a 125 μm layer of cellulose MN-300, Brinkman Instruments Inc., Westbury, N. Y.).

The cellulose layer was scored to give 20, 1 cm wide strips and the radioactive samples were applied to the origins of alternate strips using neither edge. Known mixtures of unlabeled amino acids were also applied together with the unknown or on adjacent strips. The origin spots were run to a narrow line just above their initial position with 70% ethanol. After drying, the chromatograms were developed for approximately 12 cm (3 hr) with a mixture of butanol, acetic acid, and water (4:1:5, reference 32). The chromatograms were dried and packaged with X-ray film for contact radioautography. After several days' exposure the films were developed and the chromatograms were sprayed with ninhydrin reagent and baked briefly at 100°C. The radioactive amino acids revealed on the radioautogram were identified by superimposition (co-chromatography) with the standards on the chromatogram. It was frequently possible to identify 17 amino acids in the same strip although they were not all cleanly separated. The film with alternate 1 cm strips exposed was scanned with a Schoeffel dual beam scanning densitometer (Schoeffel Instrument Corp., Westwood, N. J.). The peaks in the traces were extrapolated to a visually determined baseline and the area under them was measured with a polar planimeter to provide a semiquantitative estimate of incorporated ^{14}C which was used for comparative purposes.

Two-dimensional chromatography of photosynthetic products was carried out as previously described (8, 40), except that cellulose thin-layer plates were used instead of paper.

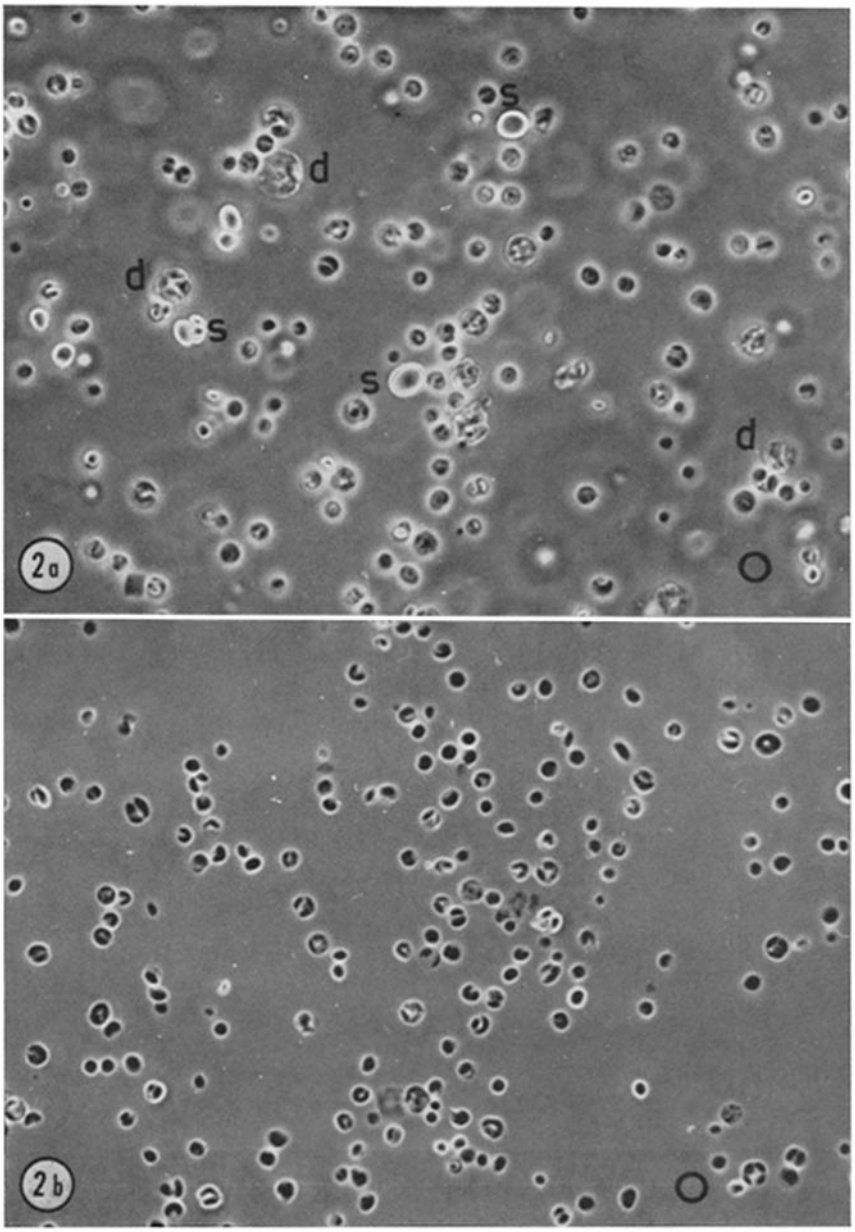
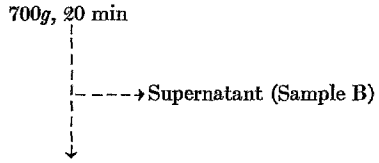


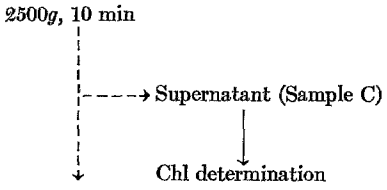
FIGURE 2 A comparison of the chloroplast isolate before and after passage through the Nucleopore filter. The starch grains (*s*) and most of the cytoplasmic droplets (*d*) seen in the upper (before) micrograph (Fig. 2 a) were removed or disrupted by filtration. The horseshoe configuration seen frequently in the chloroplasts of the lower (after) micrograph (Fig. 2 b) represent the folding of the inner lamellar apparatus within the outer membrane. The inside diameter of the circle represents 5 μ, the diameter of the membrane pore through which the chloroplasts were forced. Phase contrast photomicrographs. × 600.

FIGURE 3 Termination Procedure.

The chloroplast suspension was removed from light and kept darkened and on ice for subsequent steps. The suspension was transferred to a Pyrex tube suitable for centrifugation and subsequent sealing. (Sample A taken*)



The pellet was drained thoroughly then suspended in 8 ml of cold 80% acetone.



The pellet was washed with 70% ethanol‡ then with petroleum ether, and dried with a stream of nitrogen. 2 ml of nitrogen saturated 6 N HCl were added and tube was sealed under a partial vacuum. The pellet was hydrolyzed at 100°C for 6–16 hr. Then the seal was broken and contents were dried with a stream of nitrogen at 90°C. 1 μl of water was added for each original μg of Chl.

* 10, 25, or 50 μl samples were pipetted into 0.2 ml of 0.05 N HCl on a planchet and dried under a heat lamp.
‡ When ^{14}C amino acids were used as label, wash with cold 5% perchloric acid containing a mix of ^{12}C amino acids.

RESULTS

The Formation of Amino Acids from Carbon Dioxide

After incubations of the chloroplast isolate with $^{14}CO_2$, radioactivity is always found in the amino acids of the washed and hydrolyzed residue if the CO_2 fixation rate has been stable and linear. A high variability in the performance of the isolate observed in the earlier experiments was reduced by carefully controlling the following: (*a*) the age and condition of the cells— 1–1.5 cm exponentially growing cells provide the most consistent chloroplast isolates, (*b*) the incubation time and conditions— agitation should be minimal and the HCO_3 supply must not become limiting; and (*c*) the time of day at which the isolation is begun— there seems to be a circadian rhythm even in the isolate, and beginning the isolation 3–4 hr after "lights on" results in the most activity. Several less easily controlled variables were also encountered: (*a*) The sedimentation behavior changes, depending on the amount of stored starch in the chloroplasts, and it takes a week in darkness to "destarch" these chloroplasts. (*b*) The passage of the chloroplasts through the Nucleopore filter without damage varies not only as a result of the pressure applied and the density of the suspension, but also because both the chloroplast and, apparently, the filter pore diameters vary in different batches. (*c*) The hydrolysis time for the pellet, while easy to control, will reveal a somewhat different amino acid pattern after shorter or longer periods (9). Finally, thin-layer chromatography of crude hydrolysates is subject to many variables.

Table II and Fig. 4 summarize the amino acid labeling found in protein hydrolysates during more than 30 separate $^{14}CO_2$ fixation trials over the course of a year. It is apparent that 15 amino acids are commonly labeled and, with the exceptions of phenylalanine and hydroxyproline, incorporation into the others has been observed. The absence of radioactivity in an amino acid was usually correlated with the absence of its ninhydrin spot as well, the main exception being proline in several experiments. These problems of incomplete recovery and loss are greatly accentuated by the small protein samples available (typically 500 μg or less). Several points concerning these results should be noted. (*a*) Although serine and glycine run as one peak, there is some separation and both halves of the peak are radioactive (*b*) Arginine and lysine tend to run together when the chromatograms are heavily loaded, but in several runs they were resolved and both were labeled. (*c*) Cystine, only, is listed but any cysteine present would almost certainly have been oxidized. (*d*) Leucine and isoleucine cannot be resolved on our chromatograms, and therefore we do not know whether both are labeled. (*e*) The low frequency of proline and threonine results from destruction during hydrolysis and subsequent evaporation (reduced recovery of cystine, tyrosine, serine, and tryptophan are also likely, see reference 9). On the other hand, the hydrolysis conditions are already too mild for good

TABLE II

Incorporation of $^{14}CO_2$ into Amino Acids by Isolated Chloroplasts

Amino acid from protein hydrolysate	Experiment number*							Frequency of identification‡
	8	9	12	16	18	32	35	
Glycine + serine	+++	+++	+++	+++	+++	++	+++	100
Alanine	+++	++	++	++	++	++	++	97
Glutamic acid	+++	−	+++	+++	+++	++	++	94
Tyrosine	++	++	++	++	++	++	++	85
Aspartic acid	+++	+++	−	++	+++	++	++	75
Arginine + lysine	−	+++	++	++	+++	−	+++	62
Methionine	++	++	+	++	−	+++	++	50
Valine	−	−	−	−	−	++	+	47
Cystine	++	+++	++	−	−	+	+	38
Leucine + isoleucine	−	−	+	+	−	++	+	32
Tryptophan	−	−	−	−	+	−	+	29
Histidine	−	−	++	++	+++	−	++	25
Proline	−	−	−	−	−	++	+	6
Threonine	−	−	−	−	++	−	++	6
Phenylalanine	−	−	−	−	−	−	?§	0

− = not definitely identified or quantitation impossible.
+ = less than 5% of total amino acid label.
++ = 5%–10% of total.
+++ = 10%–20% of total.
* The conditions of the different experiments varied. As technique improved so did the number of identifiable amino acids.
‡ The per cent of 35 separate experiments where identification was definite.
§ See Fig. 4, not identified by ninhydrin, but radioactivity present in appropriate place.

release of valine and isoleucine (9). (*f*) The apparent absence of phenylalanine is disturbing; however, it was not seen as a ninhydrin spot on the chromatograms, so, either it is being obscured by other peaks as suggested in Fig 4, or it is being lost during preparation. (*g*) No standards were run for hydroxyproline, but no indication of its presence was seen with either ninhydrin or radioactivity, and it may not be present in plastid protein

Despite the technical problems inherent in hydrolyzing, recovering, and identifying amino acids from small protein samples, the evidence indicates that the chloroplast isolate is capable of incorporating $^{14}CO_2$ into all the amino acids necessary for protein synthesis.

The incorporation of $^{14}CO_2$ into amino acids could simply represent the addition of carboxyl groups or small carbon fragments rather than complete synthesis. A simple test for uniform labeling can be carried out by determining the per cent of label lost during decarboxylation (5) We have attempted to carry this out directly on the chromatograms, relying on the ninhydrin reaction to approximate a quantitative decarboxylation The chromatogram was heavily ninhydrin sprayed and baked after a first radioautogram had been exposed Then, a second radioautogram was made with an identical exposure time, and both films were developed together and compared by densitometry The results are given in Table III and Fig 5. The addition of 20 $\mu g/cm^2$ of ninhydrin should not appreciably increase the absorption of β particles, and the observed decreases are not simply proportional to the radioactivity present. Table III compares the decreases expected on the basis of complete α decarboxylation of uniformly labeled amino acids with the observed decreases after ninhydrin treatment. These results suggest that the carbon skeletons of the amino acids are being formed from CO_2 More definitive results require the complete separation of amino acids and carefully controlled decarboxylation These experiments are in progress

Further evidence that nonphotosynthetic carbon additions do not play a dominant role in the amino acid labeling is provided by the fact that the incorporation is driven by light. Table IV provides a comparison of amino acid labeling in light and

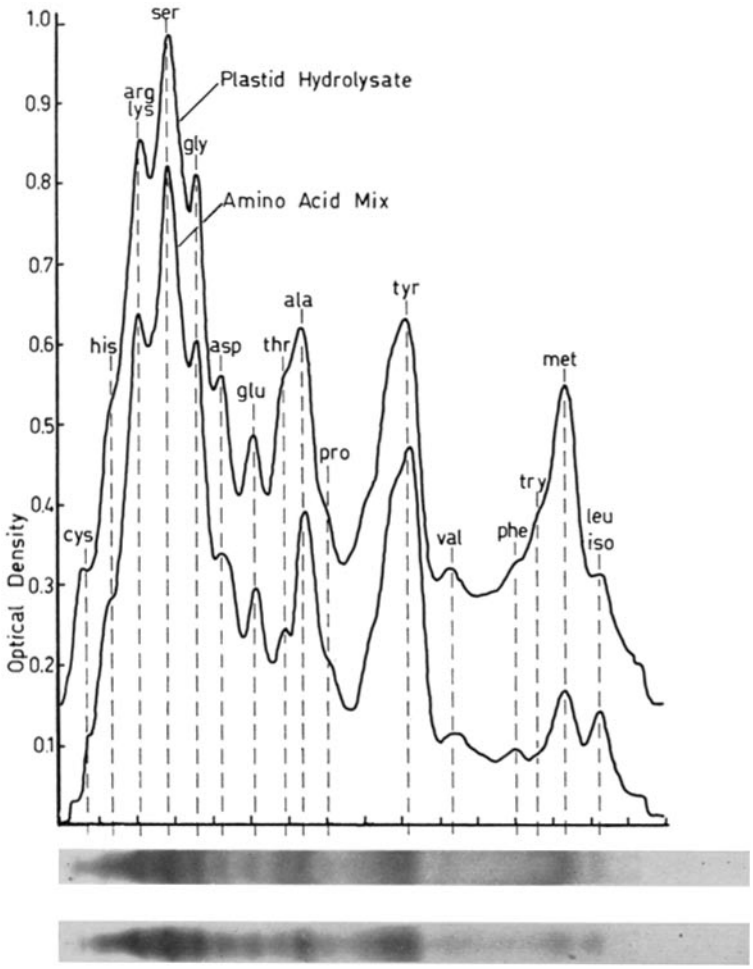


FIGURE 4 Radioautograms and densitometer traces of amino acid chromatograms. The upper trace and radioautogram represent the hydrolyzed protein of a chloroplast isolate incubated with $^{14}CO_2$ (experiment 35, Table II). The lower trace and radioautogram are of a commercial mix of ^{14}C-amino acids (Table VI). The upper trace was shifted by 0.15 OD units. The abbreviations used in this and subsequent figures are: *o*, origin; *cys*, cystine; *his*, histidine; *arg*, arginine; *lys*, lysine; *ser*, serine; *gly*, glycine; *asp*, aspartate; *glu*, glutamate; *thr*, threonine; *ala*, alanine; *pro*, proline; *tyr*, tyrosine; *val*, valine; *phe*, phenylalanine; *try*, tryptophan; *met*, methionine; *leu*, leucine; *iso*, isoleucine; *f*, front.

in darkness. Much more carbon flows into amino acids in light. Total fixation was 200-fold higher for the illuminated chloroplasts in this experiment. Thus, it appears that the chloroplasts derive the carbon skeletons of the protein amino acids from photosynthetic intermediates and products.

Performance of Chloroplasts In Vivo and In Vitro

For protein biosynthesis to occur in isolated chloroplasts, all the amino acids must be available at the same time and in adequate amounts. This has not been demonstrated conclusively by the in vitro experiments It is of course possible that the absence of some amino acids from most chromatograms represents losses due to technique, or perhaps that adequate free pools of these amino acids were already present and new synthesis was therefore repressed. Similar problems should also affect analyses of intact cells where it is certain that synthesis of plastid protein occurs. Therefore, by comparing the protein hydrolysate of the isolate

TABLE III

Results of Ninhydrin Decarboxylation

Amino acid	Radioactivity*		Per cent decrease	
	Before ninhydrin	After ninhydrin	Observed	Expected‡
Histidine + lysine	34	21	38	16
Arginine	13	11	15	16
Glycine + serine	64	42	34	60§
Glutamic acid	91	63	31	20
Alanine	18	16	11	33
Proline	4	3	25	20
Tyrosine	15	13	13	11
Methionine	21	17	19	20
Valine	4	4	0‖	25
Leucine + isoleucine	6	4.5	25	20

* Arbitrary units from areas under densitometer curves.

‡ On the assumptions of uniform labeling and complete decarboxylation of the α-carboxyl groups. Decreases greater than expected may indicate a higher than expected carboxyl label but may also be due to loss of amino acid during 100°C bake. Decreases smaller than expected are likely to indicate incomplete decarboxylation.

§ Assuming equal amounts of glycine and serine, plus loss of formaldehyde.

‖ The valine spot was small compared to the background beneath it.

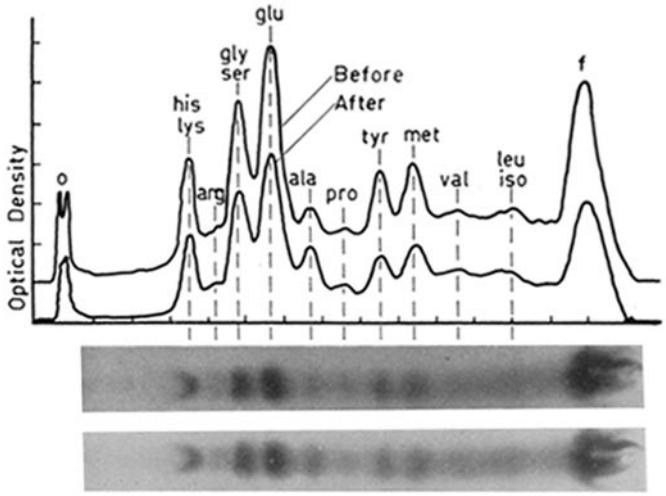


FIGURE 5 Radioautograms and densitometer traces before and after ninhydrin decarboxylation on the chromatograms. The upper trace is shifted by 0 1 OD units. The data of Table III were obtained from an expanded version of these traces On the chromatogram, the origin, some general background, and the front were ninhydrin positive. Many organic compounds are. Reaction with the ninhydrin probably accounts for their loss of radioactivity. For abbreviations, see Fig. 4.

with that of plastids isolated from sister cells after an equivalent exposure to $^{14}CO_2$ in vivo, one should be able to determine whether or not the isolate is carrying out normal protein synthesis. Such experiments have been carried out and the results from one trial are presented in Table V and Fig. 6. These data indicate that the in vitro chloroplasts produce a labeling pattern which, except for an emphasis on the basic amino acids, is similar to that obtained from the in vivo labeled chloroplasts, but the isolate channels more ^{14}C into protein This surprising fact is not entirely unprecedented since

TABLE IV

*Incorporation of $^{14}CO_2$ into Amino Acids in Light and Darkness**

Amino acid	Radioactivity‡ Light	Dark
Arginine + lysine	73	2.3
Glycine + serine	171	6.6
Glutamic acid	21	1.9
Aspartic acid	46	2.6
Alanine	15	2.9
Tyrosine	49	1.5
Methionine	18	1.4
Tryptophan	+	1.6
Valine	+	0.7
Leucine + isoleucine	+	1.1

+ = Obscured by overlapping peaks; almost certainly present in large amounts.

* Simultaneous incubations of 24 hr. The dark sample was foil wrapped.

‡ From areas under densitometer curves.

the net photosynthetic rates of the isolate exceed those seen in the intact cell in our recent experiments and those of others (16) Our previous results (7) indicate that respiratory and photorespiratory recycling of carbon is not of sufficient magnitude to account for the lower rate of fixation seen in the intact cells. Since it has also been shown that recently fixed carbon is preferentially used in the synthesis of plastid protein (6, 22, and see below), there is reason to suggest that we are dealing with a real increase in biosynthetic rate in the isolated chloroplasts. Thus, not only can the plastids carry out protein synthesis without assistance from the cytoplasm in amino acid synthesis, but the cells appear to inhibit the photosynthetic and biosynthetic activity of their chloroplasts. This should probably be viewed as a control mechanism similar to allosteric control of enzymes or control of mitochondrial respiration by the ATP:ADP + Pi ratio.

The chloroplast isolate can therefore be expected to carry out protein synthesis in a medium unsupplemented by amino acids. In order for a net increase to occur, it must be able to utilize NO_3^- and $SO_4^=$ as well as CO_2. Preliminary results suggest that it does. Investigations of nitrogen and sulfur sources as well as attempts to detect net protein synthesis are currently underway.

Utilization of Added Amino Acids

Most studies of plastid protein synthesis have been carried out using labeled amino acids as precursors and have typically supplied all the amino acids and an energy source (4, 17, 28, 42). This has also been true for previous studies with *Acetabularia* chloroplasts (11, 18). It seemed reasonable to examine the utilization of externally supplied amino acids by this highly intact isolate, and to compare its ability to incorporate them in the light and with or without ATP in darkness. It was expected that the isolates would utilize at least some amino acids, although previous experience has suggested that it did not use added ATP or various organic substrates (39) Therefore, chloroplast suspensions were provided with each ^{14}C-amino acid separately and also with a complete mix (artificial algal hydrolysate) of labeled amino acids. The latter was also presented in darkness with and without 2×10^{-4} M ATP. In another experiment the amino acids were presented in groups of four or five. The results (Table VI) were unexpected. In effect, no externally supplied amino acids were utilized to a detectable extent in any trial while sister chloroplasts (Fig. 4) actively incorporated $^{14}CO_2$ into all amino acids

The $^{14}CO_2$ provided in these experiments had a specific activity of 5 μCi/μg atom carbon, and the amino acids formed from this carbon are easily detected. These amino acids would then have a maximum specific activity ranging from 10 μCi/μmole for glycine to 45 μCi/μmole for tyrosine, while the fed amino acids had activities of 10 μCi/μmole for the single amino acids and typically 40 μCi/μg atom carbon in the mix (see Table VI). Since we could readily detect as little as 10% of the radioactivity seen in the $^{14}CO_2$ hydrolysate, the preferential utilization of recent photosynthetic products for protein synthesis rather than externally supplied amino acids is high, at least 90:1, judging from the mix. The results of the cystine and aspartate feedings confirm this judgment. Radioactivity from both of these amino acids gained access to the chloroplasts but neither was used directly for protein synthesis. Rather, their carbon skeletons were broken up and entered the carbon pool of the chloroplast. We were able to detect this radioactivity in other compounds (footnotes ** and |||, Table VI; these compounds were present in neither the original amino acid sample nor in the supernatant at the end of the incubation). Thus, our sensitivity appears to be adequate

TABLE V

*A Comparison of $^{14}CO_2$ Incorporation by Chloroplasts In Vivo and In Vitro**

Analysis of fixed carbon in cpm/μg Chl	In vivo	In vitro	In vitro/in vivo
Chloroplast suspension (sample A)	28,500	82,000	2.80
In incubation medium (sample B)	—	2,000	—
In 80% acetone extract (sample C)	21,500	46,500	2.16
Insoluble material [A − (B + C)]	7,000	33,500	4.80

Analysis of 80% acetone-soluble radioactivity‡

Compound	In vitro/in vivo
Sugar phosphates	1.82
Sucrose	2.61
Glycine + serine	2.70
Aspartic acid	1.68
Glutamic acid	1.65
Alanine	1.21
Glyceric acid	2.56
Glycolate	2.72

Analysis of radioactivity in protein hydrolysate‡,§

Compound	In vitro/in vivo‖
Cystine	6.3
Arginine + lysine	+
Glycine + serine	1.4
Aspartic acid	3.9
Glutamic acid	2.3
Alanine	2.0
Methionine	−
Proline	+
Tyrosine	7.0
Valine	1.4
Tryptophan	6.0
Leucine + isoleucine	−

* These are the results of one experiment. Cells containing an estimated 130 μg Chl were compared with chloroplasts containing 130 μg Chl isolated from sister cells. Both were incubated in 10 ml of their respective media containing 1 mg/ml carbonic anhydrase and adjusted to pH 7.50. They were exposed simultaneously to an atmosphere containing $^{14}CO_2$ at 3 mCi/mmole for 19 hr (5 light, 12 dark, 2 light). The chloroplasts were then isolated from the cells by an abbreviated version of Fig. 1 (80 μg Chl recovered) and the isolate was terminated as shown in Fig. 3 (120 μg Chl recovered). The comparisons are made per μg Chl.

‡ Ratios by densitometry of radioautograms.

§ Radioautogram presented in Fig. 6.

‖ + = not measurable in vivo; − = not measurable in vitro.

to detect any appreciable utilization of added amino acids, and recently synthesized amino acids seem to be used preferentially for protein synthesis

Our results do not contradict previous studies (8, 11) where it was reported that *Acetabularia* chloroplasts utilize ^{14}C–amino acids. These authors were able to detect the incorporation of 20 ng of total amino acid per mg of protein in the sample (Goffeau, personal communication). The technique used in our study is perhaps two orders of magnitude less sensitive, but from our $^{14}CO_2$ results we are able to say that exogenous amino acids were an insignificant source of the amino acids used for protein synthesis by the chloroplast isolate.

These results are most readily explained by the presence of a selective membrane surrounding the chloroplast. We have also frequently observed that less than 5% of the water- or acetone-soluble radioactive photosynthetic product of the chloroplast escapes to the incubation medium (see reference 8 and Table V). These soluble compounds include not only sucrose but also several amino acids. Therefore, the chloroplast appears to be a tight compartment that is quite independent of the cytoplasm in supplying its own amino acid requirements. Similar conclusions have been reached in experiments with intact algae (41) and vascular plant material (22).

DISCUSSION

These results raise several questions: (*a*) How much of the observed biosynthetic activity is due to contamination of the chloroplast isolate by other cytoplasmic components or microorganisms? (*b*)

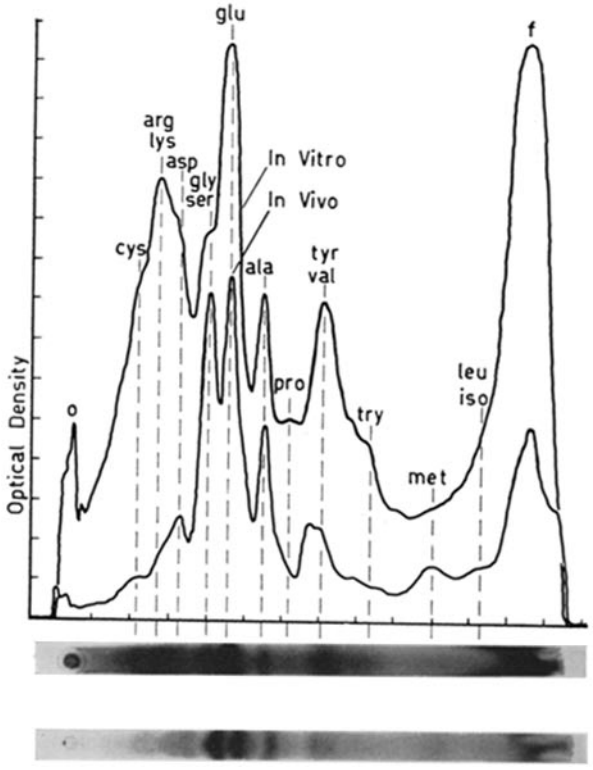


FIGURE 6 A comparison of amino acid synthesis in vivo and in vitro. These radioautograms and densitometer traces were obtained by chromatographing samples of protein hydrolysate representing equal amounts of chlorophyll. See Table V for experimental details. (There is no base line shift here.) For abbreviations, see Fig. 4.

Why do the *Acetabularia* chloroplasts behave so unlike other chloroplast isolates? (*c*) What can be said about the pathways of amino acid synthesis in these chloroplasts? (*d*) What are the implications of these findings for concepts of the interactions of chloroplasts with the rest of the cell? These questions will be considered in turn.

Contamination

Previous papers on the *Acetabularia* isolate have indicated that contamination by bacteria or mitochondria played at most a small role in the photosynthetic or respiratory activities that were studied (7, 39). For a study of amino acid and protein synthesis this evidence is not sufficient; however, the amino acid presentation experiments provide an excellent internal control. What form of contamination would use CO_2 or carbon intermediates produced by the chloroplasts for protein synthesis but would not use externally supplied amino acids? Algal contamination is not present and photosynthetic bacteria are ruled out by plate counts and by the aerobic conditions of the incubations. The only type of contamination that could give these results would be cytoplasmic elements (endoplasmic reticulum and mitochondria) in close association with the chloroplasts and mutually contained within an impervious remnant of plasma membrane or tonoplast. The characteristics of the permeability barrier seem unlikely since the intact cell will use at least some externally supplied amino acids (14). Unpublished experiments done in collaboration with R. G. S. Bidwell and his associates comparing fractions rich in cytoplasmic droplets with fractions similar to the ones used here have not shown differences in chloroplast activities. The droplet fraction simply shows considerably more evidence of Krebs' cycle and other cytoplasmic activities. Since only a fraction of the Nucleopore prepared chloroplasts are trapped with other cytoplasmic materials in small "cytoplasts" (7, and see Fig. 2), these cytoplasts

TABLE VI

Amino Acid Utilization Experiments

Amino acids presented*					Detected in protein hydrolysate of chloroplasts presented with			
					Amino acids			
Singly or in groups		As complete mix		Amino acids	Singly	In groups‡	As mix§	$^{14}CO_2$
mμmole	*μCi*	*mμmole*	*μCi*					
10	0.1	1.8	0.41	Isoleucine	−	−	−	+‖
10	0.1	0.6	0.10	Proline	−	−	−	+
10	0.1	0.8	0.18	Glutamic acid	−	−	−	+
10	0.1	1.0	0.37	Arginine	−	−	−	+¶
10	0.1	2.3	0.56	Leucine	−	−	−	+‖
10	0.1	1.4	0.48	Tyrosine	−	−	−	+
10	0.1	0.8	0.13	Threonine	−	−	−	+
10	0.1	1.0	0.15	Aspartic acid	−**	−	−	+
10	0.1	1.4	0.49	Phenylalanine	−	−	−	−‡‡
10	0.1	1.5	0.11	Glycine	−	−	−	+
10	0.1	0.1	0.02	Histidine	−	−	−	+
8	0.1	8.0	0.10	Methionine§§	−	−	−	+
10	0.1	3.4	0.53	Valine	−	−	−	+
10	0.1	2.2	0.26	Alanine	−	−	−	+
10	0.1	1.7	0.20	Serine	−	−	−	+
10	0.1	0.9	0.20	Lysine	−	−	−	+¶
1	0.2	0.8	0.20	Cystine	−‖‖	−‖‖	−	+

* Concentrations and activities per milliliter.

‡ Indicated by separating lines below.

§ Same results were obtained with mix in dark or with mix + ATP in dark.

‖, ¶ Chromatograph as one spot.

** One unidentified spot detected, not ninhydrin positive.

‡‡ May be present, see Fig. 4.

§§ Methyl ^{14}C methionine presented.

‖‖ Detected traces of leucine, glutamate, alanine, valine, and tyrosine.

would have to be many times more active than in vivo for the whole isolate to match or to better the intact cell performance on a μg chlorophyll basis. All possibility that free contaminants are responsible for the observed protein synthesis is ruled out (despite the lack of an ATP requirement, see reference 4). In fact, such contamination must be present at negligible levels or inactive.

Normalcy of Acetabularia Chloroplasts

The stability, high photosynthetic activity, and biosynthetic ability of *Acetabularia* chloroplasts have suggested to some investigators that they are highly unusual organelles (46). They do differ from higher plant plastids in several important ways. They have no proplastid stage and are green throughout the life cycle. They do not exist in a cell that ceases to grow before becoming fully functional *Acetabularia* chloroplasts are an exponenitally increasing population of functional chloroplasts during the several month growth period of the cell from which they are isolated (36, 38). Thus, biosynthesis and photosynthesis are concurrent functions in them. *Acetabularia* appears to have no peroxisome and the chloroplasts themselves carry out photorespiratory processes (7), but this is not unusual for green algae (24, 29). The isolate also appears to have an enigmatic dark respiration (7, 39), but even this has been reported in other isolates (47). In their morphology, however, they are small, unspecialized, and very ordinary looking algal chloroplasts with a pigment

complement characteristic of chlorophytes and vascular plants (30).

Beginning with the assumption that chloroplasts are possibly derived from free living ancestors, all of the properties and activities that we have described in them are reasonable. If, on the other hand, one begins with the conviction that the cell's genetic information is in its nucleus and that chloroplasts exist mostly to carry out the light reactions (the historical picture), then *Acetabularia* chloroplasts may appear anomalous.

The exceptional activity of the present isolate may be due largely to the isolation procedure. The geometry of *Acetabularia* allows the release of cytoplasm without bursting a cell that can withstand several atmospheres of pressure and forcing its cytoplasm violently through tears in the cell wall. The first cut through an *Acetabularia* cell may cause a small rise in pressure followed by a fall as its turgor is lost, but it is known (Hammerling's grafting experiments for example, reference 20) that the cell and its organelles can survive indefinitely after this operation. Subsequent cuts cause no pressure change at all, except in the path of the cut, and 10 scissor cuts through a 15 mm cell will disturb only a small fraction of the cytoplasm while releasing most of it to the homogenizing medium.

Chloroplast isolates that cannot carry out photosynthesis for reasonable times in vitro seem more suspect of preparative artifact than these. Many such chloroplast isolates are also readily permeable to exogenous substances, probably as a result of preparative damage. There seems every reason to suspect that chloroplasts, like mitochondria, should have a highly selective permeability to small organic molecules and ions. The *Acetabularia* isolate appears to have a highly selective membrane as seen here and in previous studies (39, 40). Thus, there seems to be no good reason for suspecting that *Acetabularia* chloroplasts are out of the ordinary, at least for algal chloroplasts. As to whether or not autonomy and biosynthetic activity were lost during evolution of the vascular plants, little can be said. It may be true, but then it is also true that vascular plant chloroplasts show greater activity the more gently they are isolated and when isolated from growing tissue (see reference 43).

Pathways to Amino Acids

Serine, glycine, alanine, and aspartic acid appear as free pools in the chloroplasts and are rapidly labeled from photosynthetic intermediates (8). The simplest explanation for their origin is by transamination or reductive amination of the respective α-keto acids as typical in photosynthetic tissue. The first real problem concerns glutamic acid which also forms a large, free pool (8). However, there are alternatives to the formation of α-ketoglutarate by the Krebs' cycle in plant tissue (12, 35, 41). Schemes which derive α-ketoglutarate from a condensation of glycolate or glyoxylate with pyruvate or oxaloacetate (12, 35) seem more appealing than the glyoxylate cycle or other substrate level interconversions of Krebs' cycle intermediates. Although many of the cycle intermediates are found in these chloroplasts (8), there is little evidence for their cycling (39, also see reference 5). We have attempted to determine which pathway is used to form α-ketoglutarate by isotope competition studies, using $^{14}CO_2$ and unlabeled glycolate, citrate, and several other possible intermediates, but chloroplast impermeability prevented a clear-cut result.

Little can be said concerning the pathways to the rest of the amino acids. It might be assumed that the chloroplasts retain their procaryotic characteristics and that the usual pathways are utilized. Certainly, most of the required substrates and adequate supplies of energy are available in the chloroplast, but the 50 or more required enzymes have not been demonstrated there. However, the appearance of uniformly labeled amino acids at rates comparable to those of intact cells when isolated chloroplast are fed $^{14}CO_2$ implies the presence of all of these pathways or their equivalents.

IMPLICATIONS

The inability of the isolated chloroplasts to use externally supplied amino acids plus their ability to form all of their own suggests a change in ideas of cell compartmentation which have emphasized a division of labor in the cell's intermediary metabolism (6, 31). The present evidence indicates that there are redundant systems for amino acid synthesis within the cell that interact to a very limited degree. This evidence and many data from mitochondria suggest that division of labor in the cell may be at the level of gross organelle function. The cell may see and control only a fairly simple input to and output from its organelles. Many aspects of organelle function and biosynthesis would then be adjusted and carried out *in situ*. Given origins as intracellular symbionts, such behavior seems rea-

sonable. It would enable the organelle to maintain itself despite changes in levels or types of metabolic activity in the rest of the cell. The normal patterns but higher rates for both photosynthetic and biosynthetic activity of the isolated chloroplasts imply an over-all control by the cell or its nucleus and an *in situ* control of the details of its own activities. This concept of cell organization seems more reasonable than a division of labor in intermediary metabolism which, of course, procaryotic cells can carry out very efficiently without any striking cell compartmentation, and which is carried out heterotrophically by many green plant mutants which lack functional plastids.

The degree to which these results can be generalized is not clear. There is reason to suggest that the chloroplasts of chlorophytes and vascular plants are homologous (33). Strict compartmentation of amino acid pools for protein synthesis has also been reported in vascular plants (6, 22); thus, the situation in *Acetabularia* reflects more than an ancestral condition for green plant chloroplasts. However, the evolutionary steps leading to the existence of dependent proplastid stages and the separation of growth phases from functional ones may have resulted in a much altered relationship between chloroplast and cell.

Nevertheless, until we have a better understanding of how two genetic systems within the same cell interact, a high degree of chloroplast autonomy in the control of its own activities is a reasonable working hypothesis.

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