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The Chloroplasts of Giant-Celled and Coenocytic Algae: Biochemistry and Structure

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Zusammenfassung

Alle bisher erhältlichen Ergebnisse unterstützen die Schlußfolgerung, daß Chloroplastpräparate von großzelligen und zönozytischen Algen generell robuster und fähiger sind die Kapazität zur Photosynthese für längere Zeiten beizubehalten als ähnliche Isolate von blühenden Pflanzen.

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Darüberhinaus unterscheiden sich die Photosyntheseprodukte von denen der Chloroplasten höherer Pflanzen, sowohl in den Bestandteilen, die sich innerhalb des Plastids ansammeln, als auch denen, die ins suspendierende Medium austreten. Elektronenmikroskopische Untersuchungen haben gezeigt, daß die meisten Chloroplasten in diesen Präparaten nicht frei im Medium suspendiert sind, sondern von ihm durch eine membrangebundene Struktur getrennt sind, die Zytoplasma, Vakuolenbestandteile und andere Organellen innerhalb des Raumes zwischen Chloroplast und äußerer Membran enthalten kann. Die Bezeichnung Zytoplast wurde für diese Strukturen benutzt um sie klar von Chloroplastsuspensionen zu unterscheiden, in denen die Chloroplasthülle die Barriere zwischen Organelle und suspendierendem Medium darstellt. In gewissem Sinne sind Zytoplasten Artefakte, da sie während der Isolation subzellulärer Organellen geformt werden. Dennoch ist die Fähigkeit stabile Strukturen dieser Art zu bilden wahrscheinlich ein Kennzeichen aller großzelligen Algen und Zönozyten und ein Teil des Mechanismuses, der sich entwickelt hat um Thallusverletzungen zu überleben.

Die Zytoplaststruktur selbst und im besonderen die Durchlässigkeit und Stabilität der Zytoplastmembran scheint eher als die Menge des kontaminierenden Materials, das sie umschließt, der Schlüssel zu den Unterschieden in der Stabilität und metabolischen Kapazität zu sein, die durch die Chloroplasten innerhalb des Zytoplasten zum Ausdruck kommt. Die Fähigkeit einer Behandlung mit Detergentien zu widerstehen, ist keine generelle Eigenschaft dieser Organellenisolate, doch scheint sie auf solche von Mitgliedern der Caulerpaceae und Udotaceae beschränkt zu sein, die selbst wirksame Detergentien in vivo aufweisen.

Die Fähigkeit osmotischem Schock zu widerstehen, ist, obwohl gut nachgewiesen in subzellulären Isolaten, keine generelle. Obwohl genenwärtig noch keine vollständig zufriedenstellende Erklärung für diese Eigenschaft gefunden wurde, ist die Anwesenheit gelbildender Polysaccharide, die die Organellen während ihrer Isolation umhüllen und als künstliche Zellwand agieren, eine Möglichkeit. Da diese gelbildenden Polysaccharide nicht auf zönozytische und Riesenzellalgen beschränkt sind, ist zu erwarten, daß ihre Anwesenheit Probleme bei der Untersuchung der Organellen vieler Algen bereiten könnte.

Die Schwierigkeit in der Interpretation von Ergebnissen, die mit subzellulären Isolaten dieser Algen erzielt wurden, sind zum Teil dadurch entstanden, daß sich verschiedene Autoren auf lichtmikroskopische Beobachtungen und die Anwendung von Markerenzymen verließen. Aus den Daten, die in dieser Übersicht präsentiert werden, wird klar, daß weder Lichtmikroskopie, Enzymmarker, Ferrizyanidpermeabilität noch Elektronenmikroskopie allein ausreichen, die Reinheit oder wahre Natur subzellulärer Isolate von Algen abzuschätzen. Wenn es einen Fortschritt in

der Biochemie und Physiologie dieser Organellen geben soll, und gegenwärtig sind sie immer noch ein weitgehend unerforschtes Gebiet, dann muß gute Elektronenmikroskopie der Isolate jede biochemische und physiologische Untersuchung begleiten. Ultrastrukturelle Beobachtungen von Organellen in der intakten Pflanze sind ebenfalls extrem wichtig. Es wird zugestanden, daß solche elektronenmikroskopischen Aufnahmen nicht immer einfach zu erhalten sind, doch ohne diesen kombinierten Ansatz besteht wenig Hoffnung auf Fortschritt.

Introduction

The photosynthetic organelles of eukaryotic algae show a wide range of structure and pigment composition, and although generally considered under the collective name of chloroplasts, they are probably more correctly referred to as rhodoplasts, phaeoplasts, etc., depending upon their pigment systems. The anatomy of algae, their cellular architecture and the morphology of their chloroplasts also show wide variation. The diversity expressed in the structure and in the pigment composition of algal chloroplasts might be expected to be reflected at least to some extent in their biochemistry, particularly if a polyphyletic origin is accepted for the various algal divisions.

Despite initial early success in the isolation of photosynthetically active chloroplasts from algae (Jeffrey et al., 1966; Kahn, 1966), there is relatively little information available on their biochemistry. The recent publication of a method (Klein et al., 1983a) for producing photosynthetically active chloroplasts free of major contamination from the unicellular alga *Chlamydomonas reinhardii* may stimulate fresh interest in this area. The first reports of the properties of these chloroplasts (Klein et al., 1983b) showed that in general they resembled those of higher plants in terms of their stability and their longevity, differing only in requiring a lower concentration of osmoticum in the medium for optimum stability.

The chloroplasts of coenocytic algae, which are the main subject of this review, differ from those of higher plants and of *Chlamydomonas.* Coenocytic algae are those whose cells are multinucleate and which have no cross-walls. Table I lists the occurrence of the coenocytic habit among the algae. In the plant-physiological literature the terms "siphonous" or "siphonaceous" have also been used as synonyms for coenocytic. This is not strictly correct, since siphonous algae are those which have a tubular, multinucleate thallus. Thus all siphonous algae are coenocytic (e.g. *Codium, Caulerpa*), but not all coenocytic algae are siphonous (e.g., *Griffithsia*). There are also large-celled, non-coenocytic algae such as *Acetabularia,* which in some respects resemble coenocytes. Although the coenocytic algae lack internal cell walls, some genera such as *Caulerpa*

Table I

Taxonomic position of the major algal genera mentioned in the text and other selected genera of large-celled or coenocytic algae

Taxonomy			Thallus type
CHLOROPHYTA			
Chlorococales	Characiosiphonaceae	*Characiosiphon*	C/S
Cladophorales	Cladophoraceae	*Rhizoclonium*	C/S
		Chaetomorpha	C/S
		Cladophora	C/S
Caulerpales	Derbesiaceae	*Derbesia*	C/S
	Bryopsidaceae	*Bryopsis*	C/S
	Codiaceae	*Codium*	C/S
	Udoteaceae	*Chlorodesmis*	C/S
		Udotea	C/S
		Halimeda	C/S
	Caulerpaceae	*Caulerpa*	C/S
	Dichotomosiphonaceae	*Dichotomosiphon*	C/S
	Phyllosiphonaceae	*Oestrobium*	C/S
Siphonocladales	Valoniaceae	*Valonia*	C/S
Dasycladales	Dasycladaceae	*Dasycladus*	L/S*
	Acetabulariaceae	*Acetabularia*	L/S*
CHAROPHYTA			
Charales	Characeae	*Chara*	C
		Nitella	C
XANTHOPHYTA			
Vaucheriales	Vaucheriaceae	*Vaucheria*	C/S
Mischococcales	Chlorotheciaceae	*Bumilleriopsis*	U
RHODOPHYTA			
Florideophyceae	Ceramiales	*Griffithsia*	C

C = coenocyte; **L** = large-celled (not coenocytic); **S** = siphonaceous; **U** = unicellular.
* These algae are uninucleate except during reproductive development.

have thalli several millimeters in cross-section and the lumen may be broken up by numerous projections, termed trabeculae.

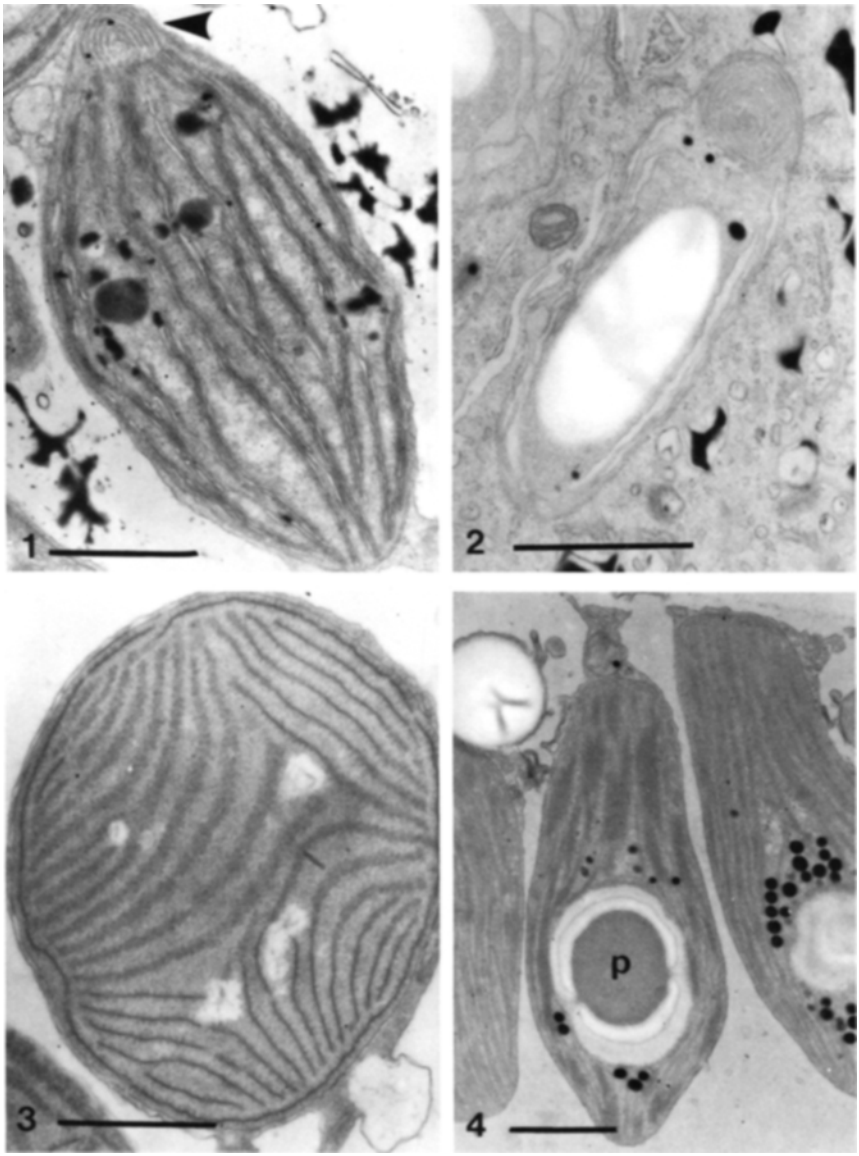
Not only is there variation in the pigmentation of algal chloroplasts, but chloroplast size and ultrastructure vary greatly (Dodge, 1973). Some of this structural variation is related to the different pigment systems found in the different algal divisions, but there are also large variations within algal divisions. This variation is also found within the giant-celled

and coenocytic algae (Calvert et al., 1976; Hara and Chihara, 1974; Hori, 1973; cf. Hori and Ueda, 1967) (Figs. 1–4). The Chlorophyta not only have a high proportion of coenocytic and large-celled genera but there is also a wide variation in chloroplast ultrastructure. For example, genera such as *Acetabularia* and *Codium* have small, simple chloroplasts with irregularly appressed thylakoids (Grant and Borowitzka, 1984; Lüttke and Bonotto, 1982) whereas the chloroplasts of the various *Caulerpa* species range from small chloroplasts without pyrenoids as in *Caulerpa filiformis,* to large pyrenoid containing chloroplasts as in *C. simpliciuscula* and *C. cactoides* (Borowitzka, 1976; Calvert et al., 1976).

The chloroplasts of the Caulerpaceae and Udoteaceae differ from other green algal chloroplasts in a number of ways. They have a concentric lamellar system (Fig. 1) at one end, called a "thylakoid organizing body" by Borowitzka and Larkum (1974a, 1974b) since it appears to be involved in the formation of thylakoids (Borowitzka, 1976). The fine structure of this body, which is an elaboration of the inner chloroplast envelope membrane, varies greatly between species (Figs. 1, 4, 7, 8) and these variations are correlated with variations in pyrenoid and starch content (Borowitzka, 1976; Calvert et al., 1976). They also have both photosynthetic plastids (i.e. chloroplasts, Fig. 1) and starch storing plastids (i.e. amyloplasts, Fig. 2), a condition known as "heteroplasty" (Feldman, 1946) (Fig. 10). Both types of plastids develop from the same kind of proplastid (Borowitzka, 1976; Borowitzka and Larkum, 1974b). These variations in chloroplast structure offer good possibilities for the study of particular aspects of chloroplast biochemistry such as the function of pyrenoids, and the differentiation and function of the thylakoids.

Interest in the giant-celled or coenocytic algae as a source of material for the isolation of chloroplasts developed as a result of two lines of thought. By the late 1960's it was possible to obtain isolated chloroplasts from spinach and pea leaves capable of fixing CO_2 at rates approaching or equal to those found in the intact leaves (Jensen and Bassham, 1966; Walker, 1967). Some workers felt that the mechanical shock necessary to shear a plant cell wall damaged chloroplasts sufficiently to prevent full expression of their in vivo potential in vitro. There was therefore uncertainty as to whether the chloroplasts, as isolated, were truly representative of chloroplasts in vivo. In particular, the failure of isolated chloroplasts which fixed CO_2 at measurable rates to form either sucrose or amino acids caused concern.

Knowledge of the transfer of metabolites between organelles within eukaryotic cells was at that time still fragmentary. The distribution of enzymes catalyzing the various steps in pathways such as that from nitrate to glutamic acid was not yet appreciated, the location of the sucrose



Figs. 1-4. Fig. 1. Mature chloroplast of *Halimeda tuna.* Note the apical thylakoid organizing body (arrow). Fig. 2. Young chloroplast of *Halimeda tuna* with large central starch grain and thylakoid organizing body. Fig. 3. Chloroplast from the red alga *Griffithsia monile* (micrograph courtesy of Dr. A. W. D. Larkum). Fig. 4. Pyrenoid containing chloroplast of the green coenocyte *Avrainvillea erecta*; (p = pyrenoid). Scale on all micrographs = 1 μm.

synthesizing system was in doubt (Bird et al., 1965, 1974) and there were reports that some isolated chloroplasts synthesized sucrose (Everson et al., 1967). A system which would allow chloroplasts to be isolated without shearing forces therefore appeared desirable. Coenocytic or large-celled algae, whose cells could be opened by simply cutting with scissors or a razor blade and the cell contents gently squeezed out and separated by differential centrifugation, offered such a system.

The first alga used for this purpose was *Acetabularia acetabulum* (Shephard et al., 1968). Most of the earlier literature uses the synonym *Acetabularia mediterranea.* Chloroplasts isolated from this species behaved differently from those of spinach and pea, thus appearing to support the hypothesis that gentle isolation techniques could yield chloroplasts active in the synthesis of sucrose and amino acids. It is now known that chloroplasts of spinach isolated by the very gentle technique of protoplast rupture (Nishimura et al., 1976) still do not produce the range of photosynthetic products characteristic of *Acetabularia* chloroplasts. The reasons for differences between higher plant chloroplasts and those isolated from this alga must be explained in other ways.

A second reason for interest in chloroplasts from giant-celled and coenocytic algae flowed from a series of observations on *Acetabularia.* It was reported that *Acetabularia* chloroplasts continued to multiply in enucleated cells, showing increases in nucleic acid and proteins under these conditions (Heilpon and Brachet, 1966; Shephard, 1965a, 1965b). At the same time there were reports that some sacoglossan molluscs (nudibranchs) retained intact chloroplasts within the cells lining their digestive diverticula for periods of up to 90 days (Kawaguti and Yamatsu, 1965; Taylor, 1968, 1970; Trench, 1969). During this time photosynthetic activity was maintained, contributing significantly to the carbon requirement of the molluscan host (Hinde and Smith, 1973, 1975). These discoveries were made in a period of resurgent interest in the theory of the endosymbiotic origin of chloroplasts (Margulis, 1970). It was logical then to propose that the chloroplasts of giant-celled and coenocytic algae might possess a greater degree of genetic autonomy than those of flowering plants, perhaps analogous to the cyanelles of *Cyanophora paradoxa* (Jaynes and Vernon, 1982; Taylor, 1970; Trench, 1982).

Reports of chloroplast division in isolated *Caulerpa sedoides* chloroplasts in culture, coupled with their capacity to maintain photosynthetic activity for long periods under those conditions were interpreted as further support for the hypothesis of autonomy (Giles and Sarafis, 1971, 1972). The DNA in the chloroplasts of *Acetabularia* has been investigated by several groups over more than a decade and the results summarized in a recent review (Lüttke and Bonotto, 1982). Aspects of this topic will be

dealt with in more detail later in this review. There is general agreement that chloroplasts in this genus do not contain the genes coding for all of the RNA and proteins within their own DNA and are dependent upon information encoded in the nuclear genome to complete their suite of structural and catalytic proteins (Apel and Schweiger, 1972, 1973; Kloppstech and Schweiger, 1973; Ibragimova et al., 1979; Astaurova et al., 1979). Therefore their existence as autonomous entities, capable of replication in the absence of the nuclear genome of the parent cells, is not possible.

No other coenocytic or large-celled alga has been studied from the aspect of nuclear/organelle interactions to the extent of *Acetabularia.* The difficulty in establishing and maintaining the coenocytes in axenic culture, free of the endosymbionts known to be present in many species (Lohr, 1975; Mariani Colombo, 1978) remains a barrier to such work being initiated. Trench and Ohlhorst (1976) studied protein synthesis in chloroplasts of *Codium fragile* in the sacoglossan mollusc, *Elysia viridis.* Although photosynthetic activity was retained during residence in the animal, and a number of proteins were synthesized, ribulose 1,5-bisphosphate carboxylase was not. Since this enzyme is essential to CO_2 fixation and is the major protein present in the chloroplast, the result suggests that these chloroplasts, in this environment, were not capable of replication and in this respect resembled those of *Acetabularia.* It is now recognized that the survival of algal chloroplasts within molluscan hosts is determined by conditions within the animal, rather than by any particular robustness of the chloroplast itself. The chloroplasts incorporated depend upon the feeding preference of the animal, and chloroplasts originating from coenocytic red algae have been observed within the animals, as have those of the non-coenocytic algae *Enteromorpha* and *Microdictyon* (Brandley, 1982; Kremer, 1977).

Data on the size of the chloroplast DNA from large-celled or coenocytic algae are very limited and allow no conclusions to be drawn except that there appears to be great variation between genera. Padmanabhan and Green (1978) found that the chloroplast DNA of *Acetabularia cliffonii* had a molecular weight of 1.52×10^9 daltons. This is larger than that of the unicellular green alga *Chlamydomonas,* which had a molecular weight of 1.9×10^8 daltons (Howell and Walker, 1976). The chloroplast genome of *Codium fragile,* at 5.6×10^7 daltons, is significantly smaller (Hedberg et al., 1981). Further data are needed to establish the range of genome sizes in algal chloroplasts and to determine whether these chloroplasts contain genes that code for a greater range of chloroplast products than do those of *Acetabularia* or of higher plants. The experimental results showing longevity, the capacity for protein synthesis and division either within the enucleated cell or in cell-free isolates do not provide evidence of genetic autonomy, although they raise a different set of questions in

the area of cell/organelle inter-relationships. In the remainder of this review, we attempt to assess critically the results obtained, in the hope that the newer techniques of biochemistry and molecular biology may be applied to the problem, which is still of fundamental interest in the field of plant biology.

Characteristics of Isolated Spinach Chloroplasts

Since repeated comparisons will be made between the algal chloroplasts and those of higher plants, it is pertinent to summarize these properties at this stage. Reviews and monographs on various aspects of this topic have appeared regularly for the past decade and detailed discussion can be found there (Barber, 1976; Halliwell, 1978; Heber, 1974; Heber and Heldt, 1981; Walker, 1976, 1980). Intact, photosynthetically active chloroplasts (Class A of Hall, 1972) fix CO_2 and evolve O_2 in light in the presence of HCO_3 at rates comparable to those observed in the intact plant; i.e. >100 μmoles CO_2 mg^{-1} chlorophyll h^{-1}. The isolated chloroplasts retain the capacity to fix CO_2 at these rates from one to three hours if held at 5°C (Giles, 1980). They have an intact outer envelope which is impermeable to ferricyanide, a classical Hill electron acceptor (Heber and Santarius, 1970). The increase in O_2 evolution resulting from ferricyanide addition is a useful means of detecting the intactness of isolated chloroplasts, although it will not detect those which have lost stromal contents but have re-sealed the damaged envelopes (Lilley et al., 1975).

Intact spinach chloroplasts export up to 90% of the carbon fixed to the suspending solution (Bassham et al., 1968). Carbon flows primarily from CO_2 to triose phosphates (Jensen and Bassham, 1966; Walker, 1967) which are then exported to the suspending medium. This translocation is effected by means of a specific phosphate translocator, sited within the inner chloroplast envelope membrane. This membrane represents the main permeability barrier to solute exchange in chloroplasts (Heber and Heldt, 1981; Heldt and Sauer, 1971). As a rule there is little synthesis of starch by isolated chloroplasts. However, starch synthesis is now known to be controlled by the stromal concentration of orthophosphate (Chen-She et al., 1975; Heldt et al., 1977) and starch synthesis can be manipulated in chloroplasts in vitro.

Sucrose and amino acids, the major carbon sinks in mesophyll leaf cells (Herold, 1980) and most green algae (Craigie, 1974) are not normally formed from $^{14}CO_2$ in isolated spinach chloroplasts. Chloroplasts will form glutamine and glutamate when supplied with the appropriate 2-oxo acids (Givan, 1975; Lea and Miflin, 1974). They can convert acetate into triglycerides (Brooks and Stumpf, 1965) and into mevalonic acid and products formed from it (Goodwin, 1971). They are also capable of car-

rying out a major part of the synthesis of the galactolipids, which are major components of the thylakoid and envelope membranes (Roughan and Slack, 1982). They will also form proteins when supplied with a variety of amino acids (Parenti and Margulis, 1967; Spencer, 1965). They reduce nitrite to ammonia (Guerrero et al., 1981) and apparently also carry out sulfate reduction (Schwenn and Trebst, 1976). These observations indicate that the higher plant chloroplast is an organelle which imports CO_2, PO_4^{3-}, NO_2^- or NH_4^+, SO_4^{2-} and a variety of oxo-acids, and exports triose-phosphate and amino acids, while retaining the capacity to synthesize some, but not all, of its protein requirements (Ellis, 1981; Jensen, 1980).

In order to isolate chloroplasts showing these properties to the fullest extent, it is essential to choose the plant species and variety with care, grow the plants under controlled conditions, and carry out the grinding of the tissue and the subsequent initial centrifugation step very rapidly at <5°C. Glassware free of detergent traces and the use of non-ionic osmotica (e.g. sucrose, sorbitol, mannitol or glycine betaine) are essential (Larkum and Wyn Jones, 1979; Walker, 1980). With the exception of the large-celled green alga *Acetabularia acetabulum* (Shephard, 1970), the unicellular green alga *Dunaliella marina* (Kombrink and Wöber, 1980) and the unicellular Xanthophyte *Bumilleriopsis* (Böger, 1969) all of which can be cultured axenically, algae used for chloroplast preparation have been taken directly from the sea and are of unknown genetic strain and growth history.

A further source of variation in algal chloroplast preparations is that the large, long cells of genera such as *Caulerpa* and *Acetabularia* generally show distinctive apico-basal organization (Bonotto et al., 1976; Dawes and Barilotti, 1969) with clear heterogeneity of their chloroplast populations (Hoursianagou-Neubrun et al., 1977). In *Acetabularia* chloroplasts, for example, the proportion of chloroplasts with DNA detectable by the use of fluorescent dyes decreases from apex to base (Lüttke and Bonotto, 1982). It is likely that similar gradients exist in other algae. These factors must be borne in mind when some of the variations found in the chloroplast preparations are considered.

There is an important exception to the previous generalizations on the properties of flowering plant chloroplasts. Albertsson and co-workers (Albertsson and Larsson, 1976; Karlstam and Albertsson, 1969; Larsson et al., 1971; Larsson and Albertsson, 1974a, 1974b) developed organelle isolation procedures using two phase polymer mixtures. With these procedures they isolated from spinach, not only Class A chloroplasts, but also chloroplasts surrounded by cytoplasm, which in turn was bound by an additional membrane. Mitochondria were also present within the cytoplasmic layer surrounding the chloroplast. These chloroplasts synthesized sucrose, malate, aspartate and alanine, as well as other amino acids,

from CO_2. Only 10% of the ^{14}C fixed accumulated as triose phosphates, in contrast to 60% in Class A chloroplasts. In addition, these chloroplasts lost only 14% of the carbon fixed to the suspending medium, while Class A chloroplasts lost 70% under the same conditions (Larsson and Albertsson, 1974b).

Chloroplasts contain their own genetic apparatus, including DNA, rRNAs, tRNAs and ribosomes, which is distinct from that of the rest of the cell (Kung, 1977; Tewari, 1971). They synthesize a range of proteins when supplied with exogenous amino acid (Ellis and Barraclough, 1978; Goffeau, 1969). Although the chloroplast DNA codes for the chloroplast rRNA and tRNA, it does not code for the complete range of mRNAs necessary to synthesize all proteins required for complete function (Parthier, 1982). The chloroplast is therefore dependent upon information encoded in the nuclear DNA (Ellis, 1981). In particular, the small subunit of ribulose 1,5-bisphosphate carboxylase, the major chloroplast protein, is coded by the nuclear DNA (Ellis and Barraclough, 1978). The more recent work in which the techniques of DNA sequencing were applied to chloroplast DNA has confirmed the dependence of the chloroplast upon the nuclear genome (Whitfield and Bottomley, 1983), although at the same time emphasizing the basically prokaryotic nature of the chloroplast protein synthesizing systems. Therefore in no sense can the chloroplast of a higher plant be considered to be genetically autonomous, and observations of chloroplast division in vitro such as those of Ridley and Leech (1970) cannot be regarded as providing models of the original chloroplast.

Methods of Isolating Chloroplasts from Giant-Celled and Coenocytic Algae

Most of the methods used to isolate chloroplasts from giant-celled and coenocytic algae depend upon mincing the cells with scissors or razor blades, either in cooled liquid medium (Bidwell et al., 1969; Cosgriff, 1973; Grant et al., 1976; Lilley and Larkum, 1981) or simply without addition of medium (Schönfeld et al., 1973). In some preparations, the algal cells have been broken in a Waring blender (Cobb, 1977; Trench et al., 1973) showing that the shear forces generated by this method did not prevent isolation of photosynthetically active chloroplasts. In our experience with *Caulerpa, Codium* and *Halimeda* chloroplasts, gentle grinding or pressure applied with rollers was essential to obtaining reasonable yields of active chloroplasts. Yields of chloroplasts from both *Caulerpa* and *Codium* could also be increased by lining the grinding vessel with cheese cloth or calico and, after mincing and rolling the tissue, wringing the brei through the cloth. The procedure used to break the smaller cells of *Bum-*

illeriopsis by Spiller and Böger (1977) differs from the above and has not generally been applied. It requires prior maceration of the cells with hemicellulase and pectinase, before disruption with glass beads in an homogenizer. The chloroplasts of *Dunaliella marina* have been isolated by lysing the cell wall-free cells of this alga through the use of DEAE-dextran (Kombrink and Wöber, 1980).

The breaking media for isolating algal chloroplasts have contained either sodium chloride (0.6–0.7 M) (Cosgriff, 1973; Grant et al., 1976; Schönfeld et al., 1973; Wright and Grant, 1978), or more commonly sugar or sugar alcohols (0.9–1.2 M) (Bidwell et al., 1969; Cobb, 1977; Lilley and Larkum, 1981) as osmotica. Chloroplasts capable of CO_2 fixation could be isolated from *Codium fragile, Bryopsis plumosa,* or *Caulerpa simpliciuscula* using either NaCl or sorbitol as osmotica (Howard, 1976), but *Bryopsis plumosa* yielded chloroplasts with higher CO_2 fixation rates when sorbitol was used in conjunction with NaCl (Wright, 1978). The same was true of *Caulerpa filiformis* (Grant and Borowitzka, 1984). On the other hand, *Acetabularia acetabulum* did not yield active chloroplasts with NaCl as the osmoticum (Shephard et al., 1968).

The use of low ionic strength media with various *Caulerpa* species resulted in viscous breis or homogenates which varied from difficult (*C. simpliciuscula*) to impossible (*C. cactoides*) to filter or centrifuge (Grant and Borowitzka, 1984) and in these species successful preparation of chloroplasts required adjustment of the ionic strength of the homogenizing medium to a level giving a freely flowing suspension. In species such as *C. peltata* this could not be achieved, even at saturating levels of salt (Grant, unpubl.)

A variety of additives to the breaking medium have been used, and of these, dithiothreitol, ascorbic acid, EDTA and bovine serum albumin appeared to give consistently improved photosynthetic rates. The addition of divalent cations, particularly Mg^{2+}, also appeared to stabilize *Bryopsis* chloroplasts (Wright, 1978), but did not give consistent results with *Caulerpa* chloroplasts (Grant and Wright, 1980). We have experimented with additions of polyvinylpyrollidine and dextran without improvement in rates of CO_2 fixation or oxygen evolution in either *Caulerpa* or *Bryopsis* chloroplasts (Howard, 1976; Wright, 1978). In general, it appears that rapid separation of the chloroplasts from the initial brei by a centrifugation step helps to achieve high activity as it does in higher plant chloroplasts. The exception is *Acetabularia* chloroplasts, where Shephard et al. (1968) used 10 and 15 minute centrifugation times and produced chloroplasts with high photosynthetic rates.

Most of the separation methods have relied solely on differential centrifugation to isolate active chloroplasts and the preparations would be expected to be contaminated to varying degrees with starch grains, mi-

tochondria, nuclei and, where field material is used, unicellular algae. These contaminants can be removed by centrifuging through layers of Ficoll® (Shephard and Levin, 1972; Wright, 1978), Percoll® (Ficoll and Percoll are registered trademarks of Pharmacia Ltd.) (Grant and Wright, 1980), sucrose (Kombrink and Wöber, 1980) or dextran (Lilley and Larkum, 1981). In a modification of their initial isolation procedure, Shephard and Levin (1972) introduced a step in which chloroplasts were forced through Nucleopore® (trademark of General Electric Corp.) filters to remove contaminating material, and obtained active and very clean preparations. Examples of these preparations are shown in Winkenbach et al. (1972); Shephard and Bidwell (1973); and Astaurova et al. (1979). This method, when applied to *Bryopsis plumosa* chloroplast isolates, yielded chloroplasts that were not aggregated and had less contamination from other organelles (Wright, 1978), but the method was unsuccessful when tested on *Caulerpa simpliciuscula* preparations (Howard, 1976). Another purification method was applied to *Codium* chloroplasts by Cobb (1977) who used the procedure introduced by Wellburn and Wellburn (1971) of gel filtration through loosely packed columns of Sephadex G-50, to remove broken and contaminated chloroplasts. This yielded chloroplasts essentially free of contamination by other organelles and showing enhanced rates of CO_2 fixation and O_2 evolution.

Storage of Chloroplasts

After isolation chloroplasts have usually been re-suspended in sucrose or sugar alcohol solutions. However, chloroplasts of *Codium vermilara* gave high rates of CO_2 fixation when stored and incubated in NaCl media (Schönfeld et al., 1973). Similar results were obtained with *Codium fragile* chloroplasts (Grant and Borowitzka, 1984) and with *Caulerpa simpliciuscula* chloroplasts (Howard, 1976).

Algal chloroplasts prepared by these methods retained their capacity to fix CO_2 for varying lengths of time. Those from *Acetabularia acetabulum* (Shephard et al., 1968), *Codium fragile* (Trench et al., 1973), and *Codium vermilara* (Schönfeld et al., 1973) all retained activity for eight hours or longer. Those from *Caulerpa simpliciuscula* and *Bryopsis plumosa* lost activity rapidly, and more than 50% of the activity had disappeared after two hours (Grant et al., 1976; Wright, 1978). While there are reports of the retention of CO_2 fixing capacity for 27 days after isolation by chloroplasts of *Caulerpa sedoides* (Giles and Sarafis, 1972) to our knowledge this result has not been confirmed in other laboratories. The unusual nature of the storage method (in which isolated chloroplasts were suspended on membrane filters in hen eggs—both white and yolk) is such that it perhaps warrants further consideration.

Properties of the Isolated Chloroplasts

Algal chloroplasts isolated by the methods described above carry out light-dependent CO_2 fixation, either with no lag or only a very short lag period after the start of illumination. They do not, in general, respond to the addition of Calvin cycle intermediates (e.g. 3-phosphoglycerate or ribose-5-phosphate) which, in higher plants, shorten the lag period before photosynthesis begins, or increase the rates of O_2 evolution and CO_2 fixation (Walker and Hill, 1967). Only in *Codium* chloroplasts was some response to these additions noted (Cobb and Rott, 1978). Since the lag period observed in chloroplast preparations of higher plants is interpreted as being due to depletion of Calvin cycle intermediates within the chloroplast, the lack of response in the algal chloroplasts suggests that there was either no depletion of the intermediates in question, or that exogenously supplied intermediates were not able to enter the chloroplast.

The rates of CO_2 fixation and O_2 evolution achieved with isolated algal chloroplasts have not, on the whole, been high relative to those obtained from higher plants. Where comparisons between chloroplasts and whole tissues have been made (Bidwell et al., 1969; Cosgriff, 1973; Grant et al., 1976; Schönfeld et al., 1973; Trench et al., 1973), the photosynthetic rates achieved with isolated chloroplasts are comparable to those of the algal thallus or cell. Moreover, since most of the material used was collected directly from the sea, and algal growth is often very seasonal, the photosynthetic rates achieved with intact algal thalli may be extremely variable. We have found with *Caulerpa simpliciuscula, Codium fragile* and *Bryopsis plumosa,* that the season during which photosynthetically active chloroplast preparations can be obtained is restricted to one or two months of the year (Grant and Borowitzka, 1984). The same is true of chloroplasts from *Griffithsia* (Lilley and Larkum, 1981). The reasons for this are presently unknown.

Another characteristic of these algal chloroplasts is that they are impermeable to ferricyanide, indicating a high proportion of intact plastids. This is confirmed by the appearance of the chloroplasts under the electron microscope (Cobb, 1977; Grant and Wright, 1980; Shephard and Bidwell, 1973; Wright, 1978). Some caution should be maintained, however, in the interpretation of ferricyanide impermeability, since in *Codium fragile* chloroplasts, at least, quite severe treatment is necessary before they show appreciable rates of ferricyanide-dependent O_2 evolution (Cobb and Rott, 1978; Grant and Borowitzka, 1984).

Products of CO_2 Fixation

There are three aspects from which the processing of CO_2 by isolated chloroplasts of large-celled and coenocytic algae may be viewed. The first

is to compare it with that observed in other chloroplast systems. The second is to compare it with the intact plants from which the chloroplasts were prepared. The third is to compare the products retained within the chloroplast to those exported to the suspending medium. As indicated in the introduction, it was the difference between the products of CO_2 fixation observed in isolated *Acetabularia* chloroplasts and those of spinach which stimulated much of the research in this field.

Wherever the kinetics of carbon flow through the products of photosynthesis in the isolated algal chloroplasts have been studied (Bidwell et al., 1970; Grant and Howard, 1980; Trench et al., 1973; Wright, 1978) the ^{14}C labelling patterns observed have been consistent with the operation of the Calvin cycle. The differences appear in the products in which the carbon is accumulated. In preparations from *Acetabularia acetabulum,* sucrose was the most heavily labelled pool within six minutes of photosynthesis. By this time both the hexose monophosphate and diphosphate and the triose phosphate pools were saturated (Bidwell et al., 1970). Sucrose, and the amino acids alanine, serine, glycine and aspartate continued to accumulate label as the period of photosynthesis increased. The rates of labelling sucrose, serine and glycine decreased during dark intervals, while alanine, aspartate and glutamate continued to accumulate ^{14}C during darkness. The amount of carbon accumulated as insoluble material was of the order of 20% of the total fixed in these experiments. In other experiments (Dodd and Bidwell, 1971) a much larger proportion (55%) of the ^{14}C accumulated in the insoluble fraction.

The pattern of ^{14}C distribution observed with *Caulerpa simpliciuscula* chloroplasts was similar to those of *A. acetabulum* in that sucrose ultimately became the most heavily labelled compound, although this occurred later, after 30 minutes of photosynthesis (Grant and Howard, 1980). The rapid labelling and larger pool sizes of hexose monophosphates were responsible for this difference, and even after 60 minutes glucose and fructose phosphates contained more ^{14}C than sucrose alone. Alanine was the most rapidly labelled amino acid, followed by glycine and serine, all of which continued to accumulate ^{14}C during the experimental period. Glutamate also accumulated label, but more slowly. Glycolic acid production was not as rapid in these chloroplasts as it was in *Acetabularia* chloroplasts. In contrast to *Acetabularia* and *Caulerpa,* chloroplasts from *Codium fragile* did not produce sucrose and produced proportionally less alanine, glycine and serine (Trench et al., 1973). The major sink for ^{14}C labelled carbon in *Codium* chloroplasts was the insoluble fraction containing an unidentified compound which was not starch. The major soluble compound labelled was glycolic acid, with hexose phosphates showing the next highest percentage of label. In *Bryopsis plumosa* chloroplasts most of the ^{14}C accumulated in a water insoluble glucan, while triose

phosphates and organic acids accounted for most of the radioactivity in the soluble fraction (Wright, 1978). Amino acids contained 9% of the total ^{14}C after 20 minutes of photosynthesis with glycine (4%), alanine (3%) and serine (1%) the main amino acids labelled. Sucrose accounted for less than 0.4% of the total ^{14}C fixed. These results show clearly that chloroplasts isolated from these four species of coenocytic algae differed from one another, as well as from chloroplast preparations of spinach and pea, in the products which they accumulated during photosynthesis.

When the ^{14}C labelling patterns obtained with chloroplast isolates were compared with those of the cells or thallus sections from which they were prepared, several other differences emerged. Isolated *Acetabularia* chloroplasts produced a range of photosynthetic products very close to the range produced by intact cells (Dodd and Bidwell, 1971; Winkenbach et al., 1972). Whole cells produced less sucrose and a greater range of other sugars than the isolated chloroplasts, but the differences were minor, the most notable being that fructosan formation did not take place in the isolated chloroplasts (Smestad et al., 1972).

This is in contrast to the results obtained with other algal chloroplasts. Those from *Caulerpa simpliciuscula* produced greater amounts of sucrose and much more hexose phosphate than the frond segments. The frond segments produced a greater range of soluble carbohydrates, and the ^{14}C in the amino acid fraction was far more evenly distributed than in the chloroplast preparation, where it was mainly in alanine (Grant et al., 1976; Howard, 1976). The apparently high activity of sucrose synthesis in the chloroplast preparations and the relatively low rates of synthesis by the frond were noted at the time, although the reasons for it were not understood. Subsequent work has shown that when frond sections are cut the rate of sucrose synthesis is depressed and is not restored until the cell has regenerated a new cell wall (Hawthorne et al., 1981). This takes at least 16 hours (Dreher et al., 1978).

Comparison between the pattern of ^{14}C fixation in whole plants and chloroplasts of *Bryopsis plumosa* has not been carried out in the same detail as in the *Caulerpa* and *Acetabularia* systems. However, the isolated *Bryopsis* chloroplasts convert more of the fixed ^{14}C into starch (57%) than the intact plant (10%), far less into soluble carbohydrate (3% as opposed to 46%) and more into amino acids (14% compared to 1%) (Wright, 1978).

A comparison of *Codium* chloroplasts with intact thalli showed that the chloroplasts produced much less sucrose, but more glycolic acid, hexose phosphate and alanine (Trench et al., 1973). A similar comparison between chloroplasts in molluscs and intact thalli showed few differences in labelling pattern (Kremer, 1978).

When the distribution of radioactivity between chloroplast and suspending medium was measured, there was usually very little release of

^{14}C from the chloroplast. In *Acetabularia* 5% of the total carbon fixed was released, compared with 30% by chloroplasts in the intact thallus (Winkenbach et al., 1972). Only 2% of the total fixed carbon was released from isolated *Codium* chloroplasts (Trench et al., 1973) and even after 24 hours only 6% of the carbon fixed during an initial 15 minute pulse was released (Hinde, 1978). However, a component known as the "host factor" isolated from molluscs with symbiotic chloroplasts stimulated the release of carbon compounds from isolated *Codium* chloroplasts (Gallop, 1974; Gallop et al., 1980). Therefore, the chloroplasts isolated from these coenocytes resemble those spinach chloroplasts isolated with membrane-bound cytoplasmic layers (Larsson and Albertsson, 1974b) and differed from chloroplasts normally isolated from higher plants. Ultrastructural studies of isolated *Codium* chloroplasts have shown many of them to be surrounded by a membrane bound cytoplasmic layer (Grant and Borowitzka, 1984), whereas the chloroplasts in the mollusc *Elysia maoria* are bound only by the chloroplast envelope (Brandley, 1981).

In contrast to the above, *Caulerpa* chloroplasts lost radioactively labelled carbon compounds rapidly. The major products released were hexose phosphates (Grant and Howard, 1980). However, the methods used in these experiments made it difficult to distinguish between material transported from the chloroplasts during the experiment and material leaked during the separation of the chloroplasts from the suspending medium.

The relatively little information available on the permeability to exogenous substrates of chloroplasts isolated from coenocytic algae suggests that it is low. The failure of Calvin cycle intermediates to stimulate photosynthesis has already been mentioned. When ^{14}C-glucose-6-phosphate and ^{14}C-3-phosphoglycerate were supplied to *Caulerpa simpliciuscula* chloroplasts the distribution of carbon observed in isolated products indicated that these compounds had not entered the Calvin cycle (Grant and Howard, 1980). In the same series of experiments the maximum rate of orthophosphate uptake observed was 1.9 μmoles mg^{-1} chlorophyll h^{-1} which was insufficient to permit CO_2 fixation to occur at more than 12 μM CO_2 mg^{-1} chlorophyll h^{-1} if all the products exported were hexose phosphate.

Acetabularia chloroplasts failed to show any increase in O_2 consumption in darkness when respiratory substrates were supplied (Shephard et al., 1968). While this was interpreted as evidence for freedom from mitochondrial contamination, subsequent work suggests that it is more likely that the substrates did not penetrate to the mitochondria present in the isolate. Although *Acetabularia* chloroplast preparations incorporated ^{14}C from $^{14}CO_2$ into proteins and amino acids, they strongly discriminated against exogenously supplied amino acids (Shephard and Levin, 1972).

The simplest interpretation of these data is that exogenous amino acids entered the chloroplasts at very low rates.

Chloroplasts from *Acetabularia cliffonii* incorporated ^{14}C labelled amino acids into a number of membrane proteins, but only into a few of the more than 50 soluble proteins detectable in chloroplast extracts separated by gel electrophoresis (Padmanabhan and Green, 1978). Although the incorporation was light-dependent, it was not dependent on the presence of a complete mixture of amino acids, presumably because of the relatively large endogenous pools of amino acid available within the chloroplast.

Chloroplasts isolated from *Caulerpa simpliciuscula* contained high levels of glutamine synthetase, glutamate synthetase and glutamate dehydrogenase (Gayler and Morgan, 1976; McKenzie et al., 1979). Furthermore, a major proportion of the amino acids generated during photosynthesis was formed within the chloroplast in this species (Smith and Gayler, 1979).

Isolated chloroplasts from enucleated *A. acetabulum* were also able to synthesize both the carotenoids and the phytol of chlorophyll from CO_2 (Moore and Shephard, 1977, 1978). The design of these experiments was such that it provided strong evidence that the entire synthesis process was within the chloroplast. A similar, though less detailed, report on the synthesis of the plastoquinones in isolated chloroplasts of this species suggests that these components too are synthesized from CO_2 in the chloroplast (Tschismadia and Moore, 1979). It has further been shown that isolated *Acetabularia* chloroplasts synthesize the apo-protein of the P-700 chlorophyll A protein complex, though not the photosystem II protein complex (Green, 1980). This synthesis was sensitive to chloramphenicol and lincomycin but not cycloheximide, thus showing that the protein was synthesized on chloroplast ribosomes and integrated into the thylakoid membranes in the absence of any cytoplasmic contribution. Although this work provided clear evidence for the synthesis of a major membrane protein de novo in isolated plastids, the author concluded that there was no evidence for translation of an unusually large number of mRNAs in comparison with higher plant chloroplasts. The evidence from Shephard's and from Moore's work points strongly to the conclusion that *Acetabularia* chloroplasts have a greater range of active enzymes than has been found in higher plants to date. If this is the situation and if there is really no greater extent of mRNA translation than in higher plants, then these proteins must be translated on the cytoplasmic ribosomal system and imported.

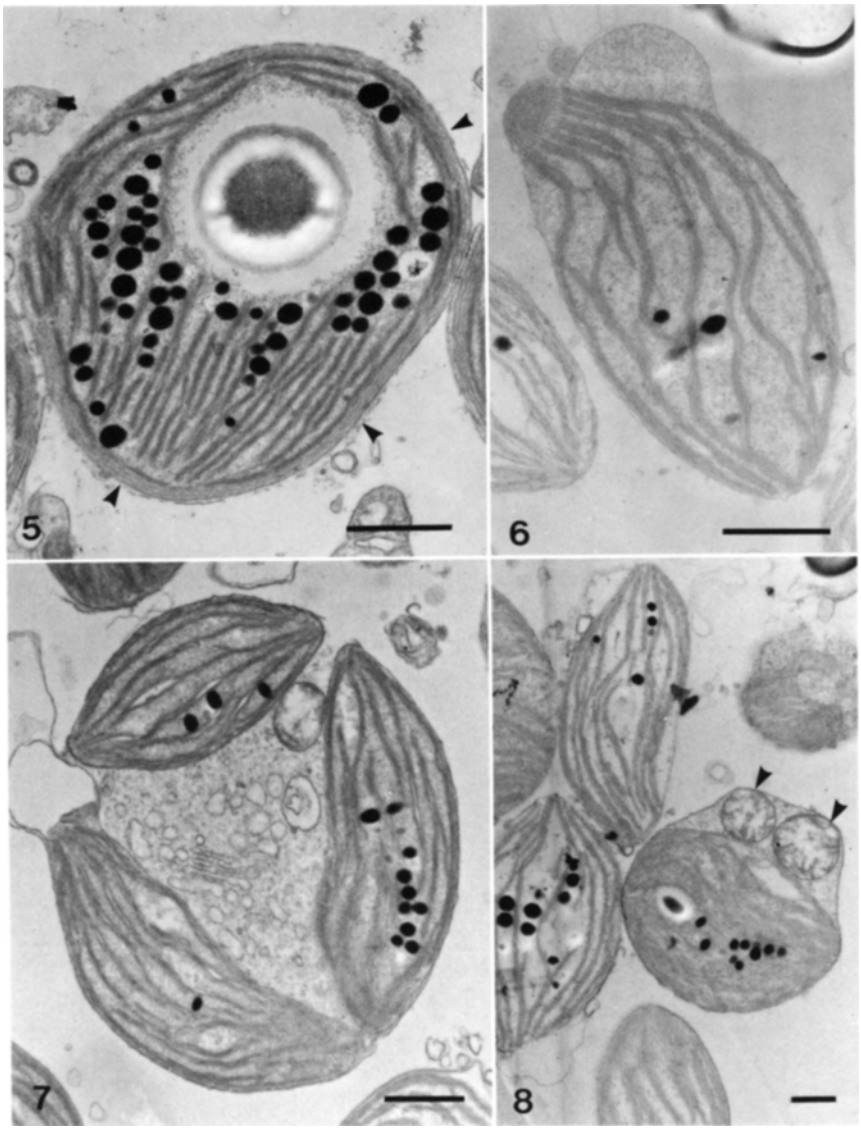
The available information suggests that while chloroplasts from these algae convert the immediate products of photosynthesis (i.e. triose and hexose phosphates) into a greater range of products than is usually seen

in isolated chloroplasts of higher plants, they release very little of this photosynthate to the suspending medium. They are not able to take up a wide variety of substrates that can be readily incorporated by spinach or pea chloroplasts. Given that in algal cells the chloroplast is the sole site of CO_2 fixation, as it is in higher plants, the low rates of transfer of fixed carbon from the chloroplast must raise doubts about the validity of these isolates as models for chloroplast function within the cell.

Structure and Purity of the Isolates

As algal chloroplasts transform the intermediates of the Calvin cycle into a greater range of products than those of spinach or pea, the enzymes necessary to catalyze these transformations must be present. Specifically, sucrose phosphate synthetase and pyruvate kinase are required for the formation of sucrose and alanine respectively. In higher plants these enzymes are located in the cytoplasm, and the development of very gentle methods of isolation of chloroplasts from spinach and pea has demonstrated that it is not damage during isolation which limits the range of products produced during photosynthesis. The key question is whether the additional capacity of the algal chloroplasts to process the products of CO_2 fixation is due to their contamination with extra-chloroplastic material, or whether it is an intrinsic property of the chloroplasts themselves. This has proved to be an extremely difficult question to probe and no definitive answer is available.

The examination of chloroplasts under the light microscope, even using phase contrast or interference contrast optics, gives no clue as to the real nature of the isolate. It will only serve to detect gross contamination by nuclei, starch grains, unicellular micro-algae and the larger bacteria. However, with the exception of the starch grains, these are generally not present in isolates, and when they are, can be readily removed by centrifuging through layers of dextran, Ficoll or Percoll. Most giant-celled and coenocytic algal chloroplast isolates are difficult to disperse, and aggregate in low ionic strength media. Under the light microscope, they appear as groups of two or three plastids, and it is not possible to distinguish whether these are aggregates of individual plastids or whether they are part of a more structured system. In the electron microscope, the chloroplasts are also often seen in groups and in the interstices between the chloroplasts, mitochondria are often observed, embedded in an ill-defined matrix which has generally been taken to be cytoplasm (Figs. 7, 8). A single membrane surrounds the entire structure (Figs. 7, 8). In isolates consisting wholly or largely of individual chloroplasts, the presence of extra-chloroplastic material, or the membrane surrounding it, is difficult to detect (Figs. 5, 6,



Figs. 5-8. Fig. 5. Isolated chloroplast of *Caulerpa simpliciuscula.* Note pyrenoid and peripheral lamellae which are part of the thylakoid organizing body (arrows). Fig. 6. Isolated chloroplast from *Caulerpa filiformis.* Fig. 7. Cytoplast in a preparation from *C. filiformis.* Note the central Golgi. Fig. 8. Cytoplasts in preparation from *C. filiformis,* one of which contains mitochondria (arrows). Scale in all micrographs = 1 μm.

8). When the plastids are suspended in a slightly hypotonic medium it is even more difficult to detect the surrounding membrane, even in high quality electron micrographs (Fig. 11).

The difficulty in determining cytoplasmic contamination and whether the chloroplasts are surrounded by an additional membrane was demonstrated by work with isolated *Codium fragile* chloroplasts (Cobb, 1977). Purification of the chloroplasts by passage through a Sephadex column significantly reduced the content of various marker enzymes for cytoplasmic, mitochondrial and microbody contamination. However, electron micrographs showed that the plastid was still surrounded by an additional membrane, Figure 5 in Cobb (1977).

In comparing the properties of chloroplast isolates it is therefore essential to distinguish between those in which the chloroplast is free in suspension (with the chloroplast envelope directly exposed to the suspending medium), and those in which the chloroplast is surrounded by an additional membrane (with a volume between it and the chloroplast proper). With the few exceptions noted earlier, higher plant leaves yield the former type of isolate, while all coenocytes and large-celled algae studied to date appear to yield the latter. The term cytoplast was introduced by Gibor (1965) to describe subcellular organelles which leaked from enucleated *Acetabularia* cells cut and suspended in hypotonic solutions. Cytoplasts differ from protoplasts which also form in *Acetabularia* (Werz, 1968) in that they lack nuclei and hence cannot survive indefinitely in vitro. The cytoplast is bound by a membrane (Gibor, 1965) and, as originally described, contains a large vacuole. The structure of chloroplasts, mitochondria and Golgi bodies present in cytoplasts has been described by Vanden Driessche et al. (1973) and contrasted with the same organelles in the intact cell.

The isolation procedures used to prepare chloroplasts from *Acetabularia* yielded preparations which were in general similar in complexity to cytoplasts (Bidwell, 1972; Winkenbach et al., 1972) except that they lacked the large vacuole. Although the nature of the chloroplast isolates was not understood in the initial studies made on *Acetabularia* chloroplasts, the problem was recognized in later work and the isolation technique modified to disrupt the chloroplast aggregates and remove most of the extra-chloroplastic material (Shephard and Levin, 1972). There was remarkably little difference in properties of chloroplasts prepared by this improved method, and those used in the initial studies (Winkenbach et al., 1972). The conclusion reached was that in the highly purified preparations any contaminating cytoplasm or membrane contributed little beyond stability to the preparation (Shephard and Bidwell, 1973). Attempts made to estimate the amount of cytoplasmic material present using enzyme markers

were unsuccessful because of the low levels of suitable enzyme activity present in the cell extracts (Winkenbach et al., 1972).

Chloroplasts prepared from *Caulerpa simpliciuscula* also proved difficult to free from contamination. *Caulerpa simpliciuscula* chloroplasts are surrounded by multiple membranes, the outermost of which is the chloroplast envelope (Borowitzka, 1976), and in isolated chloroplasts it was difficult to determine whether the isolated chloroplasts and adhering material were surrounded by a further membrane (Grant and Wright, 1980; Grant et al., 1976). Examination of a large number of preparations under the electron microscope showed that the majority of the chloroplasts in the isolate were in the form of cytoplasts. In these preparations, the amount of contamination was estimated indirectly by use of marker enzymes. Chloroplast preparations were found to contain 10% of the mitochondria and 6% of the cytoplasmic material present in the original homogenate (Grant and Wright, 1980). Minor variations in isolation technique resulted in significant variations in these values, showing that it is not valid to assume uniformity between different preparations (Gayler and MacKenzie, unpubl.).

Attempts were made to estimate the amount of cytoplasmic material present in *Caulerpa* preparations by using cytoplasmic ribosomal RNA as a marker (Wright, 1978). This method has been used to estimate the extent of cytoplasmic contamination in other chloroplast preparations (Bird et al., 1974; Price et al., 1973). It suffers from a number of difficulties, including the assumption that cytoplasmic ribosomes are not preferentially associated with subcellular organelles—an assumption that is certainly unjustified in some algae (Gibbs, 1962; Oliveira and Bisalputra, 1973). The results obtained using this method showed that while the cytoplasmic ribosomal RNA accounted for between 72 and 75% of all RNA extracted from both pea leaves and *Caulerpa* during chloroplast isolation, the *Caulerpa* chloroplast pellet contained almost three times as much cytoplasmic rRNA as the pea chloroplast pellet (60.6% and 23.1% of the total, respectively). Furthermore, all attempts to decrease the level of cytoplasmic rRNA by methods that normally remove ribosomes bound to the surface of sub-cellular organelles (such as washing with EDTA, KCl and Puromycin) failed (Wright, 1978). These same experiments showed that only 16% of the *Caulerpa* cytoplasmic rRNA could be found in the supernatant remaining after centrifugation at 15,000 *g* for 15 minutes. This fraction would be expected to contain most of the free cytoplasmic ribosomes and rough endoplasmic reticulum.

Chloroplasts isolated from *Codium vermilara* (Ben-Shaul et al., 1975) and from *C. fragile* (Cobb, 1977) also showed evidence of contamination, although it was reported that this could be removed in part by washing (Ben-Shaul et al., 1974) or by passage through Sephadex columns (Cobb,

1977). From the limited work carried out on *Bryopsis plumosa* chloroplasts it appears that these were also surrounded by cytoplasmic material, but that the total amount of this cytoplasmic contaminant was relatively small (Wright, 1978).

The presence of non-chloroplast material in all of these chloroplast isolates makes it extremely difficult to evaluate the results of metabolic experiments. However, the results showed that the metabolic capacity of the isolated algal chloroplasts was not directly related to the amount of contamination and therefore that contamination alone is not likely to be responsible for the range of products produced.

There are two possible explanations for this greater diversity of metabolic products observed in the chloroplasts of coenocytic and giant-celled algae. The products of CO_2 fixation observed in vitro may be a genuine reflection of the situation in vivo and therefore these chloroplasts carry out many metabolic steps which in higher plants are located in other subcellular compartments. This means that they must contain a more extensive suite of enzymes synthesized de novo from mRNAs coded for by the chloroplast DNA, from mRNAs imported as primary transcripts from the nucleus, or imported as polypeptide chains. As indicated earlier, the evidence available supports the view that a considerable proportion of the information required for synthesis of both structural and catalytic proteins is encoded on the nuclear DNA, so the question of complete genetic autonomy does not arise. This does not, however, exclude the possibility that chloroplasts of these species have more complex genomes and code for a greater range of proteins than has been found in higher plants.

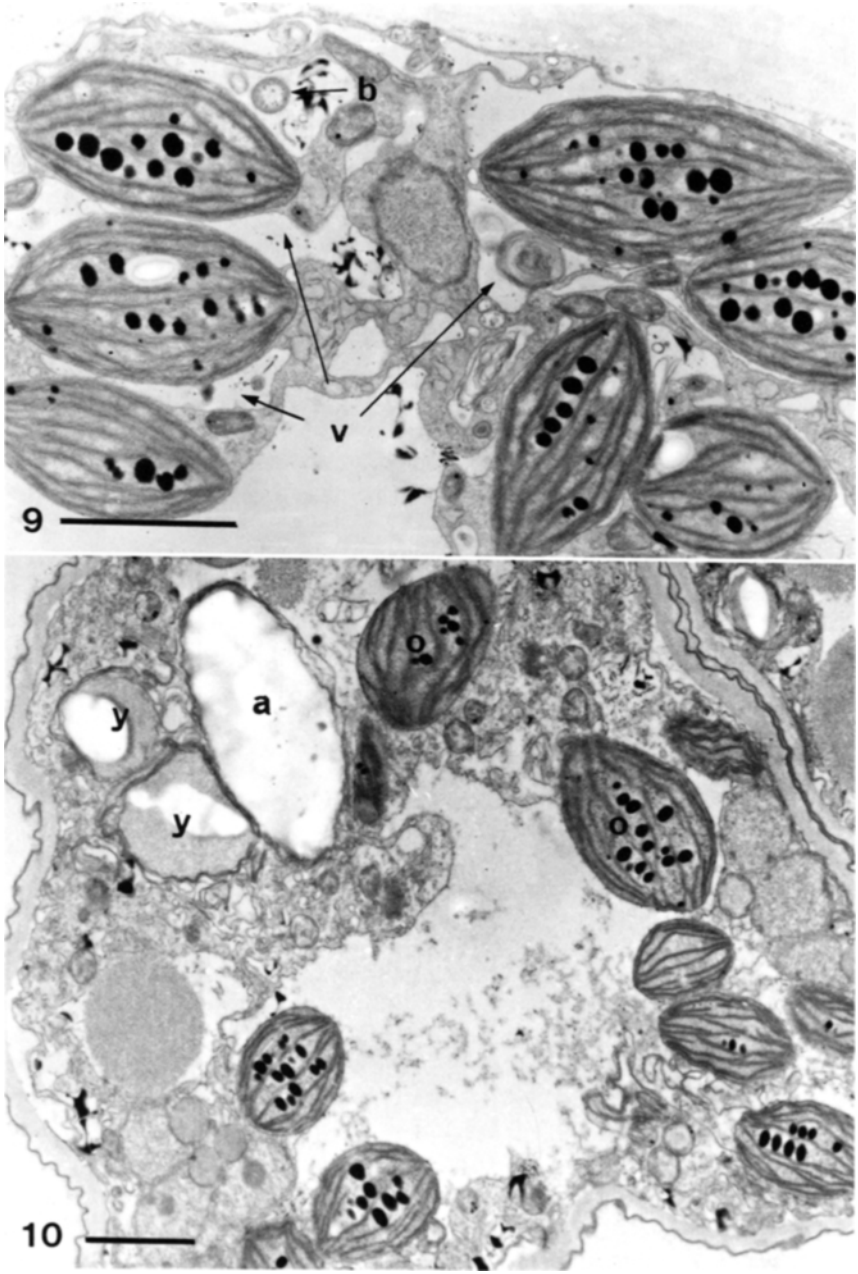
The details of the structure and complexity of the *Acetabularia* chloroplast genome have been discussed in a recent review (Lüttke and Bonotto, 1982). The average size of the chloroplast DNA has been reported to be $1.52 \pm 0.25 \times 10^9$ daltons, compared to 2×10^8 daltons from the chloroplast DNA of *Chlamydomonas,* and there were on average three genomes per plastid (Padmanabhan and Green, 1978). These authors suggested that this unusually large size might reflect a greater retention of the genome of the original endosymbiont, even though full autonomy had been lost (Green et al., 1977). The DNA content fluctuates widely between individual plastids (Coleman, 1979; Lüttke and Bonotto, 1981; Woodcock and Bogorad, 1970) making it difficult to draw firm conclusions on the basis of genome size alone. A recent study which showed continuous plastid DNA replication during growth was considered to be an indication of prokaryote-like rather than eukaryote-like control of DNA synthesis (Coleman and Maquire, 1982). It is clear that the question of the actual coding capacity of the chloroplast genome in these algae, as well as that of the coenocytes, is ready for exploration by the restriction endonuclease

method of sequencing coupled with examination of in vitro translation of gene products, as discussed by Whitfield and Bottomly (1983).

A second possibility is that the structure of the isolated organelles is not representative of those in the cell and the plastid and contaminating material are surrounded by an additional membrane. Such a membrane would limit the diffusion or transport of metabolites. This would result in a buildup of metabolite concentrations within both the chloroplast and the extra-chloroplastic space. Thus a relatively small amount of cytoplasmic contamination would be sufficient to catalyze the formation of compounds such as sucrose and amino acids at the rates observed, which were low in absolute terms. The existence of a small extraplastidic compartment could also affect the pH gradients across the chloroplast envelope, and thus the stromal pH, which in turn would affect CO_2 fixation and metabolism (e.g. Anderson, 1979; Werdan et al., 1975). Until it is possible to prepare chloroplasts from these algae free of these additional membranes and to compare these with the preparations isolated to date, it is not possible to distinguish between these alternatives. Efforts to achieve this have not yet met with success.

In normal, functioning cells neither chloroplasts nor other sub-cellular organelles are present in the form of cytoplasts, although they may be appressed to either the plasmalemma or the tonoplast (Borowitzka and Larkum, 1977; Dawes and Rhamstine, 1967; Hoursiangou-Neubrun and Puiseux-Dao, 1974) (Figs. 9, 10). To this extent these aggregates in chloroplast preparations are artifacts of the isolation procedures used. However, it has also been shown that when the cells of these coenocytes are wounded, either by cutting or even by penetration with a needle, the cytoplasmic organization changes rapidly (Dreher et al., 1978; Lohr, 1975; Menzel, 1982). Cytoplasmic continuity was lost in the vicinity of the wound and the entire cytoplasmic mass formed aggregates. These aggregates ranged in size and complexity from extremely simple structures containing single plastids or mitochondria, to those containing several plastids, mitochondria, Golgi bodies and sections of vacuole. The larger and more complex structures were found furthest from the wound site. The inclusion of a nucleus in the aggregates qualifies them to be described as protoplasts, which could have the capacity to reform a new cell wall and eventually a new cell. This has in fact been observed in *Bryopsis* (MacRaild, 1974) but not in *Caulerpa.* It should be noted that the formation of the motile gametes of coenocytic algae such as *Caulerpa, Codium* and *Halimeda* and the large-celled alga *Acetabularia* also proceeds by the intracellular formation of protoplasts which develop flagella and which are eventually released from the cell (Borowitzka, unpubl.; Meinesz, 1981; Valet, 1969).

There is a progression in complexity from the smallest cytoplast to a fully developed protoplast. The relative size distribution in an isolate will



Figs. 9 and 10. Fig. 9. Section through thallus of *Caulerpa scalpelliformis*. Note the highly dissected cytoplasm interdigitated by vacuole (v). Fig. 10. Section through thallus of *Halimeda cylindracea* showing both young (y), and mature (o) plastids and an amyloplast (a). Scale in all micrographs = 2 μm.

depend on the method used to disrupt the cell. Cytoplast formation, in this respect, is a normal aspect of the biology of coenocytic and large-celled algae, and probably evolved as a means of dealing with the problems posed by damage in a large cell which has no transverse cell walls.

The origin of the cytoplast membrane is not known. The limiting membrane of protoplasts prepared by enzymic digestion of plant cell walls is the plasmalemma. The origin of the limiting membrane of cytoplasts prepared by the cutting of large-celled algae is uncertain. Electron micrographs and light microscopic observations of the cells of large-celled and coenocytic algae show that the cytoplasm is often much dissected by the vacuolar or tonoplast membrane, and although it is difficult to estimate, the total tonoplast membrane surface area is much greater than the plasmalemma surface area (Figs. 9, 10). It therefore seems likely that the cytoplast membranes have a tonoplast origin. Data on the penetration of NH_4^+ and NO_3^- into the cytoplasts of *Acetabularia* support this view (Grant et al., 1972). It is also possible that the cytoplast membrane forms de novo from components of the plasmalemma, tonoplast and endoplasmic reticulum. "Self-assembling" lipids are known (Sitte, 1981) and it is possible that following the massive disruption to cell organization due to the crushing and grinding of large-celled algae, various membrane and free lipids together with proteins form a "new" membrane around protoplasmic droplets. Protoplasmic droplets of *Nitella* have been shown to form a surface membrane (Inoue et al., 1973; Ueda et al., 1973, 1974) and the properties of this membrane varied with the ionic composition and temperature of the bathing solution. The resealing of damaged chloroplast envelope membranes (Lilley et al., 1975) also represents a "self-assembly" process.

The cytoplast membrane of *Caulerpa* and *Codium* differs in its staining properties in hypotonic solutions from other cell membranes (Grant and Borowitzka, 1984) suggesting a chemical composition higher in protein content or saturated lipids than commonly found. Whatever the origin of the cytoplast membrane, it is clear that it does not exist as such in the unwounded cell, and that it forms as a result of cell disruption. We believe that the capacity of large-celled algae and coenocytes to form such membranes in turn determines their capacity to form cytoplasts. For example, chloroplasts isolated from the large-celled red alga *Griffithsia monilis* do not appear to form cytoplasts (Lilley and Larkum, 1981). This alga also shows no wound healing response.

Experimental results suggest that it is the cytoplast membrane surrounding the isolated organelles, rather than contamination per se, which is responsible for many of the properties of chloroplasts prepared from giant-celled and coenocytic algae. In this context, it should be noted that the formation of cytoplasts also takes place in non-coenocytic species, for

example, in the brown alga *Fucus serratus* in which the isolated chloroplasts are present within cytoplasts (Nordhorn et al., 1976).

Robustness of Isolated Chloroplasts

Reports of unusual stability of giant-celled and coenocytic algal chloroplast isolates cover four aspects: (1) their capacity to remain photosynthetically active for very long periods when compared with chloroplasts isolated from other species, (2) the capacity to retain integrity in the presence of high concentrations of NaCl, (3) the ability to resist osmotic shock, and (4) the capacity to resist dissolution by non-ionic detergents at concentrations which lead to disruption of most lipid bilayer membranes. Details of the retention of chloroplast activity after isolation have been introduced earlier, and we shall now discuss this longevity in terms of a cytoplast structure.

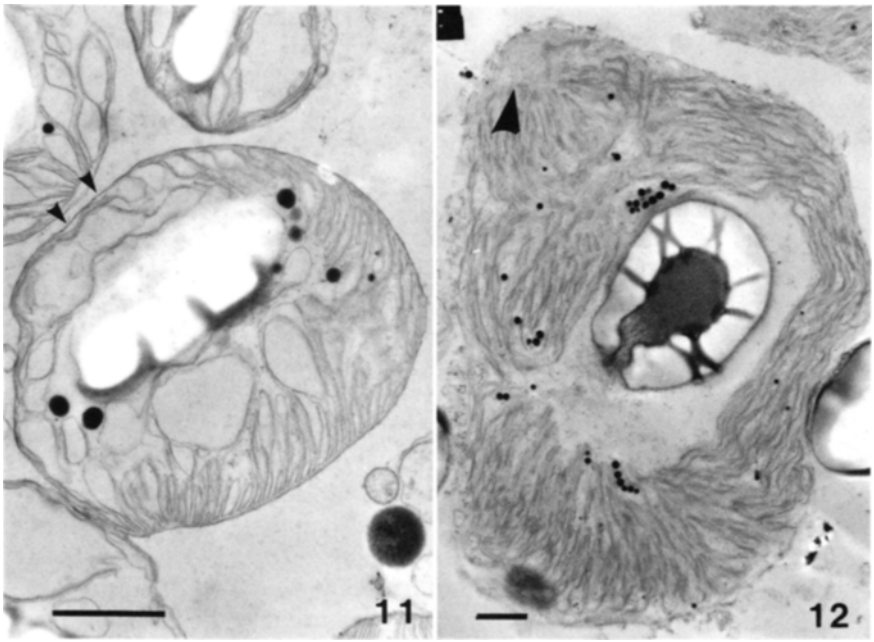
As there is strong evidence against genetic autonomy, after isolation one is therefore dealing with survival of chloroplasts and not with reproduction. There is no doubt, however, that chloroplasts isolated from these algae do survive for extremely long periods. On the basis of what is known of the biochemistry of spinach and pea chloroplasts, their capacity to carry out CO_2 fixation requires that the soluble enzymes (stromal compartment) be retained at high concentration in proximity to the thylakoid membranes. In chloroplast suspensions this usually means that the chloroplast envelope must be intact, although high rates of CO_2 fixation have been obtained with envelope-free chloroplasts under suitable conditions (Delaney and Walker, 1976). The envelope of isolated spinach or pea chloroplasts is readily removed by high concentrations of ions, particularly Na^+. It will also reseal after rupture (Lilley et al., 1975) and withstand some variation in tonicity of the suspending medium before fragmentation as indicated by the degree of swelling shown when the osmolarity of the suspending solution is decreased from 0.33 to 0.16 M sorbitol (Heber and Heldt, 1981; Heldt and Sauer, 1971). However, it is generally agreed that breakdown of the chloroplast envelope, followed by dilution of stromal enzymes and intermediates, is responsible for the gradual loss of CO_2-fixation activity in isolated spinach chloroplasts.

If in coenocytic algal chloroplasts the organelle is enveloped in a cytoplast membrane, with the space between this cytoplast membrane and the outer chloroplast envelope containing some of the original cytoplasmic proteins, then this would provide a stabilizing environment. If an equally high protein concentration could be maintained in solution in the absence of a cytoplast structure, then it might be possible to extend the life of any isolated organelle. This was the concept behind the original experiments on survival of coenocytic algal chloroplasts reported by Giles and Sarafis

(1971, 1972; Sarafis, pers. comm.). The cytoplast membrane is normally formed in giant-celled and coenocytic algae in response to wounding, thus it must be a stable structure capable of retaining its integrity in high ionic strength media such as sea water. Although direct evidence is lacking, the cytoplast membrane is probably unique in its properties and responsible for at least one aspect of the robustness of the chloroplasts isolated from coenocytic algae. In any future studies of cytoplasts, the formation, organization and composition of this membrane must have a high priority.

The capacity of these chloroplasts to resist osmotic shock, however, cannot be explained solely by the presence of cytoplast membranes. Evidence of resistance to osmotic shock has been obtained from two types of experiment. It was shown that *Caulerpa sedoides* chloroplasts did not swell and burst when suspended in distilled water (Giles and Sarafis, 1974). We have found the same to be true of isolated chloroplasts of *C. cactoides* (Fig. 12) and *C. filiformis* (Grant and Borowitzka, 1984) and true, in part, of chloroplasts of *C. simpliciuscula* (Grant et al., 1976). The ultrastructure of chloroplasts of *Caulerpa filiformis* suspended in 50 mM buffer showed them to have lost some, but not all of their stroma and only some very slight swelling of the thylakoids was evident (Grant and Borowitzka, 1984). Chloroplasts of *Codium fragile* treated in the same way lost more of their stroma and showed greater thylakoid swelling, but generally maintained intact envelopes (Fig. 11). None of these changes could be detected in the light microscope, giving a clear warning of the dangers of attempting to assess the effects of any particular procedure on the structure of these chloroplasts by light microscopy alone.

Another approach has been to study the effects of change in the osmotic pressure of the suspending solution on rates of CO_2 fixation, ferricyanide permeability, O_2 evolution and stromal enzyme leakage (Cobb and Rott, 1978; Grant and Borowitzka, 1984; Grant et al., 1976; Schönfeld et al., 1973). Where this has been done a slightly different picture emerges. When the sucrose concentration in the suspending medium of *Codium fragile* chloroplasts was reduced from 0.8 to 0.1 M, the CO_2 fixation rate fell from 35 to 5 μM mg^{-1} chlorophyll h^{-1} (Cobb and Rott, 1978). Oxygen evolution declined from 45 to 7 μM mg^{-1} chlorophyll h^{-1} (Grant and Borowitzka, 1984) over the range of 1300 to 100 mOsmolar. At the same time the permeability of the chloroplasts to ferricyanide remained very low until the osmolarity of the suspending solution fell to 300 mOsmolar. Even when the chloroplasts were suspended in solutions of 100 mOsmolar the rate of ferricyanide-dependent O_2 evolution was less than 50% that obtained with sonicated chloroplasts. If the osmolarity was reduced to 300 mOsmolar and then was increased to 1200 mOsmolar the rate of CO_2-dependent O_2 evolution was restored to $\frac{2}{3}$ of its original rate (Grant and Borowitzka, 1984). Since the chloroplasts used in Cobb and Rott's



Figs. 11 and 12. Isolated chloroplasts suspended in isolation medium containing no osmoticum. Fig. 11. *Codium fragile.* On very careful examination the cytoplast membrane (arrows) can be distinguished from the chloroplast envelope membranes. Most of the stroma has been lost and the thylakoids are swollen. Fig. 12. *Caulerpa cactoides.* There is little loss of stroma and little thylakoid swelling. Note the thylakoid organizing body (arrow). Scale on both micrographs = 1 μm.

(1978) work were initially isolated in 9% sucrose (250 mOsmolar) the results obtained in the two laboratories are comparable.

The chloroplasts of two species of *Caulerpa* (*C. simpliciuscula* and *C. filiformis*) behaved slightly differently. Chloroplasts from *C. simpliciuscula* could tolerate a reduction of osmotic strength from 900 to 450 mOsmolar but lost 60% of their activity when the osmolarity was further reduced from 450 to 220 mOsmolar. The ferricyanide-dependent O_2 evolution rate increased from 0 to 12 μM mg^{-1} chlorophyll h^{-1} at this stage. Further reduction of the osmolarity to 100 mOsmolar caused loss of all O_2 evolution and a light-dependent O_2 uptake of 12 μM O_2 mg^{-1} chlorophyll h^{-1} was observed, while the ferricyanide-dependent rate increased to 32 μM O_2 mg^{-1} chlorophyll h^{-1} (Grant et al., 1976). Restoration of the chloroplasts to 1200 mOsmolar did not restore their capacity to fix CO_2 or evolve O_2. Osmotic shock however did not result in the loss of large amounts of stromal material, as measured by the release of stromal

enzymes ribulose 1,5-bisphosphate carboxylase and glucose-6-phosphate dehydrogenase. Pea chloroplasts lost 60% of their stromal content under the same conditions (Wright and Grant, 1978). Chloroplasts from *C. filiformis* lost their capacity for CO_2-dependent O_2 evolution as the osmolarity was reduced from 1200 to 600 mOsmolar and ferricyanide-dependent O_2 evolution increased rapidly as the osmotic pressure fell from 600 to 100 mOsmolar. These chloroplasts did not regain their activity if resuspended in 1200 mOsmolar solution (Grant and Borowitzka, 1984). It is therefore clear that *Codium* and *Caulerpa* chloroplasts are relatively insensitive to the osmotic potential of their environment, and that *Codium* chloroplasts are the more resistant.

Two possible explanations can be proposed for this behavior. The chloroplast envelope may be almost impermeable to water, and hence changes in external osmotic pressures are not reflected by water movement into the chloroplast. This seems improbable, both from known membrane properties, and because there is temporary or permanent loss of photosynthetic activity following reduction in the osmotic potential of the suspending medium. Alternatively, the isolated chloroplast could be surrounded by some structure with a tensile strength able to resist the pressures generated by an osmotic differential of at least 0.9 molar. While there is no evidence to date that such a structure exists, it has been mentioned earlier that many of the coenocytic algae contain gel or gel-forming materials within their vacuoles. These gels are able to withstand considerable deformation and do not readily dissolve or disperse in water (Dreher et al., 1982). Furthermore, these materials, which are sulfated xylo-mannogalactans (Dreher et al., 1982; Hawthorne, 1980), would not be stained by the normal stains used in electron microscopy (Oliveira et al., 1980). It is therefore possible that thin layers of gel could surround the chloroplasts and escape detection under the electron microscope. Their presence would impart tensile strength to the isolated organelle. It would be immaterial whether the gel layer was inside the cytoplast space or external to it. Some indications of a gel layer of this type coating cytoplasts have been observed in histochemical studies of both *Codium* and *Caulerpa* chloroplasts (Borowitzka, unpubl.; Grant and Borowitzka, 1984).

The final distinctive characteristic of these coenocytic algal chloroplasts is their capacity to withstand attack by detergents, specifically Triton X-100 and sodium dodecyl sulfate. To date this property has only been reported in chloroplasts isolated from the Caulerpaceae and Udoteaceae (Borowitzka, 1976; Giles, 1980; Giles and Sarafis, 1974; Wright and Grant, 1978). Resistance to Triton X-100 was not found in *Acetabularia* chloroplasts (Shephard et al., 1968; Winkenbach et al., 1972), though stability in 1% Triton X-100 under hypertonic conditions was reported by Goffeau (1969); *Codium* chloroplasts were also lysed by detergent (Cobb, 1977).

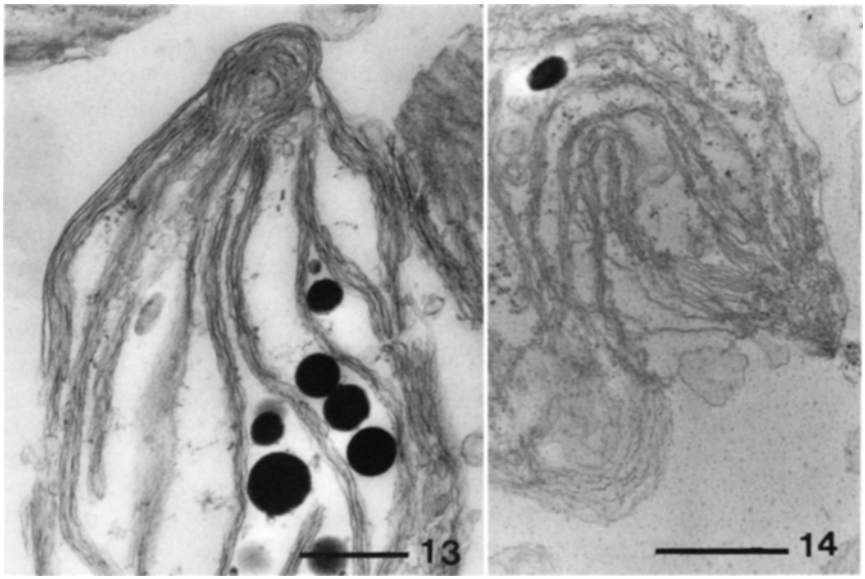
In their study of the effect of detergent on *C. simpliciuscula* chloroplasts Wright and Grant (1978) found that if the chloroplasts were exposed to 0.1% Teric-10 (equivalent to Triton X-100) there was disruption of the chloroplast and dissolution of the thylakoids only if the chloroplasts were suspended in hypotonic media, suggesting that the variation found in *Acetabularia* preparations was a result of tonicity of the medium. Wright and Grant proposed that the site of detergent resistance was located in either the chloroplast or cytoplast envelope. This was consistent with a demonstration of a sodium dodecyl sulfate and Triton X-100 resistant structure in *C. cactoides* and *C. sedoides* chloroplasts (Giles and Sarafis, 1974).

All members of the Caulerpaceae and Udoteaceae contain a multiple membrane structure called the thylakoid organizing body which is apparently an elaboration of the inner chloroplast envelope and which is usually located at one end of the plastid (Borowitzka, 1976; Borowitzka and Larkum, 1974a; Calvert et al., 1976; Hori and Ueda, 1967) (Fig. 12). In some species of *Caulerpa,* notably *C. simpliciuscula, C. cactoides* and *C. sedoides,* extensions of this thylakoid organizing body may surround completely the periphery of the chloroplast (Borowitzka, 1976; Calvert et al., 1976) (Fig. 5). It was inferred that it was this structure which gave the chloroplasts their resistance to detergents and also to osmotic shock.

Triton X-100 was ineffective in removing the material which adheres to the chloroplast (Wright, 1978). In *Caulerpa filiformis,* a *Caulerpa* species without the peripheral lamellae of the thylakoid organizing body, 0.01% (w/v) Triton X-100 removed the cytoplast membrane and caused partial loss of the chloroplast envelope but did not solubilize the thylakoids or all of the material adhering to the chloroplast (Fig. 13). At 0.1% Triton X-100 the chloroplast envelope, together with parts of the thylakoid organizing body were solubilized, but the thylakoids were still clearly recognizable (Fig. 14). Similar results were also obtained with *C. cactoides* chloroplasts. These latter preparations were so heavily contaminated with cytoplasmic and other material that more detailed observations of the chloroplast and cytoplast membranes were not possible (Grant and Borowitzka, 1984).

The chloroplasts of *Codium fragile,* which do not have a thylakoid organizing body, were equally resistant to solubilization by Triton X-100 when viewed under the electron microscope, but detergent treatment inhibited O_2 evolution at 0.01%. *Caulerpa* required 0.1% or greater concentration to achieve the same result (Grant and Borowitzka, 1984).

It has been established that the chloroplasts of *Caulerpa* and *Codium* are both resistant to detergent solubilization, although the site of this resistance has not been determined. It is clearly not the cytoplast membrane, nor does it appear to be the thylakoid organizing body. The fact



Figs. 13 and 14. Effects of Triton X-100 on isolated chloroplasts from *Caulerpa filiformis*. Fig. 13. Preparation treated with 0.01% (v/v) Triton X-100. Fig. 14. Preparation treated with 0.1% (v/v) Triton X-100. Although the plastid structure is greatly disrupted the thylakoids and the thylakoid organizing body can be distinguished. Scale on both micrographs = 1 μm.

that such resistance to surface active agents exists is in itself unusual. Caulerpalean and related algae contain a number of surface active compounds such as rhipocephalin and caulerpeyne which have been proposed to act as antifeedants (Sun and Fenical, 1979; Targett and McConnell, 1982; Wells and Barrow, 1979) and which have been shown to be toxic to many organisms. It is possible that the chloroplasts in these algae are exposed to these surface active compounds in vivo and have evolved resistance to them.

Summary and Conclusions

All of the available evidence supports the conclusion that chloroplast preparations from large-celled and coenocytic algae are generally more robust and able to retain the capacity to carry out photosynthesis for longer periods than similar isolates from flowering plants. Furthermore, the products of photosynthesis differ from those of higher plant chloroplasts, both in the compounds which accumulate within the plastid, and those leaked to the suspending medium. Electron microscope studies have

shown that most of the chloroplasts in these preparations are not freely suspended in the medium, but are separated from it by a membrane-bound structure which may contain cytoplasm, vacuolar contents and other organelles within the space between the chloroplast and the outer membrane. The term cytoplast has been used for these structures to distinguish them clearly from chloroplast suspensions in which the chloroplast envelope is the barrier between organelle and suspending medium. In one sense, cytoplasts are artifacts, inasmuch as they are formed during the isolation of the subcellular organelles. However, the capacity to form stable structures of this sort is probably a characteristic of all large-celled algae and coenocytes and is part of the mechanism evolved to survive thallus wounding.

The cytoplast structure itself, and in particular the permeability and stability of the cytoplast membrane rather than the amount of contaminating material which it encloses, appears to be the key to the differences in stability and metabolic capacity expressed by the chloroplasts within the cytoplast. The capacity to resist detergent treatment is not a general property of these organelle isolates, but appears restricted to those from members of the Caulerpaceae and Udoteaceae, which contain powerful detergents in vivo.

The capacity to resist osmotic shock, although well established in the subcellular isolates from several species of these algae, is not general. While there is at present no completely satisfactory explanation for this property, the presence of gel-forming polysaccharides that coat the organelles during their isolation and act as an artificial cell wall is a possibility. Since these gel-forming polysaccharides are not restricted to coenocytic and giant-celled algae, their presence could be expected to create problems in the study of organelles from many algae.

The difficulties in the interpretation of results obtained with subcellular isolates from these algae have, in part, arisen from various investigators' reliance on observations made at the level of light microscopy and by the use of marker enzymes. From the data presented in this review, it is clear that neither light microscopy, enzyme markers, ferricyanide permeability nor electron microscopy alone is sufficient to assess either the purity or true nature of subcellular isolates from algae. If there is to be progress in the biochemistry and physiology of these organelles, and they still represent a largely unexplored field, good quality electron micrography of the isolates must accompany any biochemical or physiological studies. Ultrastructural observations of the organelles within the intact plant are also extremely important. It is recognized that such electron micrographs are not always easy to obtain, but without this combined approach, there is little hope of progress.

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