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# Specific Deficit in the Synthesis of 6-Sulfoquinovsyl Diglyceride in *Chlorella pyrenoidosa*[1]

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It was found that when *Chlorella pyrenoidosa* was grown on cysteine as the sole sulfur source, it lost the ability to grow photoautotrophically. When grown in the presence of glucose, cysteine-grown cells displayed a doubling time in the light or dark of 45 h, which is identical to that of cells grown on glucose and $SO_4$ in the dark. This suggests that cells grown on cysteine as sole sulfur source can only grow heterotrophically. In support of this hypothesis, it was found that cysteine-grown cells were defective both in vivo and in vitro in $CO_2$ fixation, although $O_2$ evolution in such cells was normal. Assays of the enzymes of the Calvin cycle indicated that the deficit in $CO_2$ fixation could be ascribed to a lowered phosphoribulokinase activity. A total lipid analysis of *Chlorella* grown on cysteine revealed that such cells showed a 100-fold deficiency in the purportedly chloroplast-associated 6-sulfoquinovsyl diglyceride. This agrees with earlier reports that cysteine could not serve as a precursor of sulfolipid in *Chlorella*. No other polar lipid was affected. Large amounts of triglyceride, however, were found in cysteine-grown cells. The biosynthesis of triglyceride provides a means of utilizing reduced nicotinamide adenine dinucleotide reducing equivalents not being used for $CO_2$ fixation.

The polar lipids of biological membranes exhibit considerable chemical heterogeneity (36), suggesting a possible heterogeneity of function. It has, however, been extremely difficult to unequivocally demonstrate specific roles for specific lipids.

A reasonable approach to demonstrating specificity is a reconstitution experiment (12). It has been possible to reconstitute activity of several membrane enzymes only when a particular lipid is added to the preparation. Recently, however, studies by Higashi and Strominger (19) and Sandermann (33) on *Staphylococcus aureus* polyisoprenol kinase have demonstrated that alteration in conditions of reconstitution and assay can reveal activation by lipids previously thought incapable of activation.

Other biochemical methods, such as "specific" membrane lipid perturbation or co-purification of a membrane protein with a particular lipid, which are sometimes used to demonstrate specific protein-lipid interaction, are also equivocal because of the now obvious dynamic nature of membrane lipid distribution (13).

Clearly, it would be highly desirable to complement the biochemical studies with in vivo experiments designed to specifically delete one or more lipids and examine the physiological and biochemical effects of such deletions. This has been attempted with microorganisms by mutagenesis, and in all cases the mutation has been lethal (6, 9, 10, 25). For studies of lipid specificity in enzyme function, what would be most desirable would be a specific loss of a lipid without loss in the capacity of the organism to divide.

As this report will show, deletion of 6-sulfoquinovsyl diglyceride (29) from *Chlorella pyrenoidosa* is possible without prevention of growth of this organism. This is because this sulfolipid is found only in the chloroplast (11) of algae (as well as higher plants). Since *Chlorella* can be grown heterotrophically on glucose, the expected chloroplast dysfunction upon loss of this lipid would not prevent cell growth. Since the sulfolipid is only a minor component of the total chloroplast lipid – about 3% (27) – it was anticipated that at least partial chloroplast synthesis would take place in the absence of synthesis of this lipid.

In fact, this article will demonstrate that it is possible to obtain a specific deficiency of 6-sulfoquinovsyl diglyceride in *Chlorella* chloroplasts without substantially perturbing membrane chemistry or structure. This is done by growing cells on cysteine as sole sulfur source. Defi-

[1] Contribution no. 222 from the Eleanor Roosevelt Institute for Cancer Research, Denver, Colo.

ciency in sulfolipid results since cysteine is not a precursor for sulfolipid in this organism (28).

## MATERIALS AND METHODS

**Growth of cells.** Slants of *C. pyrenoidosa* (Emerson strain 3) were kindly provided by Jerome Schiff. Cells were grown on the sulfur-free medium described by Hodson et al. (20) except that $NaNO_3$ was used instead of $KNO_3$. Either $K_2SO_4$ or cysteine hydrochloride (filter sterilized) was added to a final concentration of 0.5 mM. The medium was supplemented with 0.5% glucose for some experiments.

Cells were grown with shaking at 25°C under 4,000 lux from daylight fluorescent lamps. Cells were grown in the dark by wrapping the flasks in black electrical tape. Growth rates were monitored by measuring apparent absorbance with a Klett-Summerson colorimeter fitted with a green (560 nm) filter (20).

**Conversion of $^{35}S$-labeled precursors to sulfolipid.** Cells were grown on glucose-salts medium with $^{35}S$-labeled $SO_4^{2-}$ or cysteine as sole sulfur source at a specific activity in each case of 2 $\mu$Ci/$\mu$mol. Radioactive cysteine was supplied as labeled cystine (Amersham) in a large molar excess of unlabeled cysteine. Cells were grown to saturation on these precursors, harvested by centrifugation at 2,000 $\times$ *g* for 10 min, and washed three times with cold salts medium. Total cell lipids were extracted by a modification of the procedure of Bligh and Dyer (8). Cells were taken up in 2.4 ml of 0.1 N HCl and extracted overnight with 9 ml of MeOH-$CHCl_3$ (2:1) in a screw-cap test tube. The residue was removed by centrifugation, and 3 ml each of $CHCl_3$ and 1 M $H_2SO_4$ was added to the supernatant. The organic phase was removed by aspiration and evaporated under a stream of nitrogen.

The residue obtained was then applied to a silicic acid thin-layer chromatography (TLC) plate that was developed with $CHCl_3$/MeOH-water (65:25:4 by volume) and visualized by a Packard model 7201 radio-TLC scanner. Cells grown on labeled sulfur give two symmetrical peaks on this system: a large one with an $R_f$ of 0.32 (sulfolipid B) and a smaller one with an $R_f$ of 0.21 (sulfolipid A).

**Lipid analysis.** For total lipid analysis, cells were grown in the light on medium supplemented with [$^{14}C$]sodium acetate (New England Nuclear, Boston, Mass.; specific activity, 10 $\mu$Ci/mmol). After seven generations the cells were harvested by centrifugation, washed two times with 0.05 $Na_2PO_4$ buffer, pH 7.0, and suspended in 2.4 ml of this same buffer in a test tube with a Teflon-lined screw cap. Nine milliliters of MeOH-$CHCl_3$ (2:1) was added (8), the tubes were tightly sealed, and the tops were wrapped in Teflon tape. The samples were placed in a rotating test tube rack at 37°C and extracted for 1 week. At this time, 3 ml each of $CHCl_3$ and saturated NaCl was added, and the bottom phase was removed by aspiration (8).

The total extracted lipids were separated by the procedure of Renkonnen and Bloch (32) on a 1-g silicic acid (Bio-Sil A, Bio-Rad Laboratories, Richmond, Calif.) column into neutral, glycolipid, and phospholipid fractions. These lipid classes were further fractioned by TLC on commercial silicic acid plates. (EM Reagents Division, Brinkmann Instruments, Inc., Westbury, N.Y.). Neutral lipids were developed first in diethylether-benzene-ethanol-acetic acid (40:50:2:0.2 by volume) and then in ether-hexane (6:94 by volume) as described by Freeman and West (14). Glycolipids were developed in $CHCl_3$-MeOH-water (65:25:4 by volume; 32), and phospholipids were developed in $CHCl_3$-MeOH-isopropylamine-$NH_4OH$ (65:35:0.5:0.5 by volume; 1). Spots were revealed by radioscanning and identified by cochromatography with bona-fide mass standards visualized by charring with phosphomolybdic acid (31).

**In vivo studies of photosynthesis.** Carbon dioxide fixation was measured as described by Goodenough et al. (15). Freshly grown cells were resuspended in growth medium (no glucose) at a concentration equivalent to 12.5 $\mu$g of chlorophyll per ml. Two milliliters of cell suspension was placed in a water-cooled reaction chamber at 24°C illuminated by a 150-W projector floodlamp at an intensity of 3,600 foot-candles. After 5 min of exposure for equilibration, 0.2 ml of $NaH^{14}CO_3$ (specific activity, 8.4 $\mu$Ci/$\mu$mol at a concentration of 1 mM) was added. Aliquots of 0.1 ml were withdrawn at 1, 2, 3, 4, and 5 min and placed in scintillation vials containing 0.1 ml of a 4:1 mixture of concentrated HCl and glacial acetic acid. Controls were run in the dark. The samples were evaporated to dryness under an infrared lamp, and then 0.2 ml of 0.05 N phosphate buffer (pH 7), 1 ml of Bio-Solv (Beckman Instruments, Inc., Fullerton, Calif.), and 15 ml of scintillation fluid were added. The amount of $^{14}CO_2$ fixed was then determined with a liquid scintillation counter.

Oxygen evolution was determined as described by Armstrong et al. (2). Freshly grown cells were resuspended in growth medium (without glucose) containing 2.5 mM $NaHCO_3$ at a cell titer equivalent to 12.5 $\mu$g of chlorophyll per ml. The cells were equilibrated with stirring in the light for 1 h in a thermostated reaction vessel at 24°C. Illumination was provided by a 1,000-W projector lamp at an intensity of 10,000 foot-candles. Oxygen evolved was measured with a Yellow Springs Instrument Co. oxygen monitor (model 53) fitted with a Clark-type electrode.

**Enzyme assays.** Cells were resuspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.0, containing 0.01 M dithiothreitol and broken by three passes through a French pressure cell at 24,000 lb/in$^2$. Whole cells were removed by a 4-min centrifugation at 480 $\times$ *g* at 0°C.

The rate of conversion of ribose-5-phosphate to 3-phosphoglyceric acid was determined as described by Levine and Togasaki (24). The assay mixture contained, in a final volume of 1 ml: 100 $\mu$mol of Tris, pH 7.5; 5 $\mu$mol of $MgCl_2$; 10 $\mu$mol of glutathione; 20 $\mu$mol of $KHCO_3$; 5 $\mu$mol of neutralized adenosine triphosphate (ATP); 5 $\mu$mol of ribose-5-phosphate; 4.5 $\mu$mol of $NaHCO_3$; 0.5 $\mu$mol of $NaH^{14}CO_3$ (specific activity, 50 mCi/mmol); and between 60 and 90 $\mu$g of protein. At various times, 0.2-ml aliquots were withdrawn and added to scintillation vials containing 0.1 ml of 5:1 concentrated HCl and acetic acid. The samples were evaporated to dryness under a heat lamp, and the amount of $CO_2$ fixed was quantitated by liquid scintillation counting. The reaction

was linear with time for 15 min at 30°C.

The phosphopentoisomerase reaction was assayed as described by Moll and Levine (26). Cell extracts, prepared as described above, were centrifuged for 90 min at 138,000 × *g*. The resulting supernatant was dialyzed for 2 h at 4°C against 0.05 M, Tris buffer, pH 7.0, containing 0.01 M dithiothreitol. The assay mix contained, in a final volume of 1.0 ml: 0.2 mg of protein; 100 μmol of Tris buffer, pH 7.5; 5 μmol of $MgCl_2$; and 10 μmol of ribose-5-phosphate. The reaction was run at 30°C. Fifty-microliter aliquots were removed after 3, 5, 7, and 9 min and added to 3 ml of 70% $H_2SO_4$ followed immediately by the addition of 0.1 ml of 1.5% cysteine hydrochloride and 0.1 ml of 0.12% carbazole in ethanol. These samples were then heated at 37°C for 30 min, and the optical density at 540 nm was determined with a Zeiss spectrophotometer.

Phosphoribulokinase was assayed in dialyzed 480 × *g* supernatants by radiochromatographic analysis of the product. The assay mix contained, in a final volume of 1 ml: 100 μmol of Tris buffer, pH 7.5; 1 μmol of dithiothreitol; 5 μmol of $MgCl_2$; 0.5 μmol of ATP labeled at the γ position with $^{32}P$ (New England Nuclear; specific activity, 20 mCi/μmol); 0.5 μmol of ribulose-5-phosphate; and 1.25 mg of protein. After the reaction proceeded at 30°C for 5 min, a 0.1-ml aliquot was removed and added to 0.1 ml of 10% trichloroacetic acid in a 0.5-ml ignition tube. The precipitate was removed by centrifugation, and 25 μl of the supernatant was applied to commercial 0.1-mm cellulose TLC plates (Brinkmann Instruments). These plates were developed with methanol-88% formic acid-water (80:15:5 by volume; 4) and visualized by a radio-TLC scanner. On this system, ribulose diphosphate migrates with an $R_f$ of 0.45. Unreacted ATP remains at the origin, whereas labeled inorganic phosphate runs near the solvent front. The radioactive bands were scraped from the plate into scintillation vials and ribulose diphosphate synthesis was quantitated by liquid scintillation counting.

Ribulose diphosphate carboxylase was assayed in dialyzed 480 × *g* supernatants. The assay mix contained, in a final volume of 1.0 ml: 0.2 mg of protein; 100 μmol of Tris buffer, pH 7.5; 5 μmol of $MgCl_2$; 10 μmol of reduced glutathione; 0.1 μmol of ribulose-1,5-diphosphate; 20 μmol of $KHCO_3$; and 0.5 μmol of $NaH^{14}CO_3$ (specific activity, 50 mCi/mmol). Aliquots of 0.2 ml were taken every 2 min for 8 min and added to 0.1 ml of glacial acetic acid in a scintillation vial. The samples were evaporated to dryness under a heat lamp, and the amount of $CO_2$ fixed was quantitated by liquid scintillation counting.

**Electron microscopy.** Cells were fixed in glutaraldehyde and osmium tetroxide, embedded in Araldite 6005 (Ciba), Epon 812 (Shell), and DDSA (Allied Chemical), and stained with lead citrate and uranyl acetate as described by Goodenough and Levine (17). Electron microscopy was performed with an HU-11C Hitachi electron microscope.

**Protein and chlorophyll determinations.** Protein was determined by the biuret method (23) except that the trichloroacetic acid-precipitated protein was washed three times with cold acetone and dried under a stream of nitrogen before the color reagent was added. This served to remove interfering pigments.

Chlorophyll determinations were carried out on material extracted from cells with 80% dimethyl formamide by the method of McKinney as described by Arnon (3).

**Liquid scintillation counting.** Counting was performed on a Beckman liquid scintillation spectrometer (model LS-230, Beckman Instruments, Inc.) with scintillation fluid consisting of 15.1 g of PPO (2,5-diphenyloxazole) per gallon (3.78 liters) of toluene.

**Substrates.** Radioactive [$^{35}S$]cystine (9 mCi/mmol), $^{35}SO_4^{2-}$ (100 mCi/mmol), γ-[$^{32}P$]ATP (10 Ci/mmol); [1,2-$^{14}C$]sodium acetate (50 mCi/mmol), and $NaH^{14}CO_3$ (50 mCi/mmol) were obtained from New England Nuclear. Ribose-5-phosphate, ribulose-5-phosphate, and ribulose-1,5-diphosphate were obtained from Sigma Chemical Co., St. Louis, Mo.

## RESULTS

**Growth properties of cells grown on cysteine as sulfur source.** Doubling times of *Chlorella* grown with cysteine or $SO_4^{2-}$ as sole sulfur source were obtained by monitoring turbidity as described in Materials and Methods. *Chlorella* grown on cysteine as sole sulfur source exhibited the growth properties expected of a cell growing heterotrophically (Table 1). Cysteine-supplemented cultures demonstrated an absolute growth requirement for glucose, and cultures supplemented with cysteine plus glucose exhibited a doubling time that was about twice that of cultures supplemented with $SO_4^{2-}$ plus glucose. However, cultures supplemented with cysteine showed doubling times similar to those of cultures supplemented with $SO_4^{2-}$ when these cultures were grown in the dark. Experiment 4, reported in Table 1, shows that cysteine was not giving these results through cytotoxicity.

The heterotrophic growth of *Chlorella* when cysteine was sole sulfur source indicates a malfunction in photosynthesis.

**Deficiency of sulfolipid in *Chlorella* grown on cysteine as sole sulfur source.** In an effort to confirm (28) that cysteine cannot provide

TABLE 1. *Growth rate of Chlorella on cysteine for sulfate as sulfur sources*

| Expt no. | Sulfur source | Glucose? | Light? | Doubling time (h) |
|---|---|---|---|---|
| 1 | Cysteine | No | Yes | ∞ |
| | $SO_4^{2-}$ | No | Yes | 20 |
| 2 | Cysteine | Yes | Yes | 42 |
| | $SO_4^{2-}$ | Yes | Yes | 22 |
| 3 | Cysteine | Yes | No | 46 |
| | $SO_4^{2-}$ | Yes | No | 43 |
| 4 | $K_2SO_4$ + Cysteine | No | Yes | 20 |

sulfur for sulfolipid synthesis, 200-ml cultures of *Chlorella* were grown to saturation in the light in medium supplemented with either $^{35}S$-labeled cysteine or $SO_4^{2-}$ as sole sulfur source. Incorporation of these precursors into sulfolipid was determined as described in Materials and Methods. Cysteine was only about 1% as effective as $SO_4^{2-}$ as a precursor for sulfolipid (Table 2).

To conclusively demonstrate the expected deficiency of sulfolipid in cells with cysteine as sole sulfur source, cells were grown with [$^{14}C$]acetate supplements in cultures containing either $SO_4^{2-}$ or cysteine. Sulfolipid was then isolated from these cells by silicic acid column chromatography followed by silicic acid TLC as described in Materials and Methods. Studies with $^{35}S$-labeled sulfolipid revealed that the column procedure used split the total sulfolipid into two fractions. Some 40% of the total sulfolipid applied eluted with the glycolipids (fraction II), and the remainder eluted with the phospholipids (fraction III). TLC analysis of these fractions (Fig. 1) showed the sulfolipid peaks to be virtually absent.

**Photosynthesis in *Chlorella* grown on cysteine as sole sulfur source.** The rates of $CO_2$ fixation and oxygen evolution were examined in vivo with the expectation that photosynthesis would be defective in sulfolipid-deficient cells. Cysteine-grown cells fixed $CO_2$ at a substantially lower rate than did cells grown on $SO_4^{2-}$ (Table 3). As has previously been reported by Latzko and Gibbs (22), growth of cells on glucose supplements also depressed the rate of $CO_2$ fixation. If the low level of $CO_2$ fixation observed in cysteine-grown cells is the maximum activity that such cells can manifest, the obligate heterotrophy of cysteine-grown cells can be explained. The data show that photoautotrophic $SO_4^{2-}$-grown cells had 15 times as much $CO_2$-fixing activity as did cysteine-grown cells. It is interesting to observe that although $CO_2$ fixation was strikingly different in $SO_4^{2-}$- and cysteine-grown cells, oxygen evolution was not. This requires that cysteine-grown *Chlorella* turn over photosynthetically produced reducing equivalents by some mechanism other than $CO_2$ fixation. As will be discussed, this mechanism may be fatty acid synthesis.

The observation of defective $CO_2$ fixation suggests that cysteine-grown cells are defective in one or more enzymes of the Calvin cycle in which ribose-5-phosphate is converted to phosphoglyceraldehyde. The data in Table 4 confirm this hypothesis and demonstrate that the inability of cysteine-grown cells to fix $CO_2$ can be ascribed to a deficit in phosphoribulokinase activity.

**Lipid and chlorophyll composition of *Chlorella* grown on cysteine as sole sulfur source.** The data shown in Fig. 1 and Table 5 indicate little alteration in the relative levels of polar lipids other than the sulfolipid and are consistent with the hypothesis that the deficiency is specific. On the other hand, these data also demonstrate an obvious difference in neutral lipid content between cysteine- and $SO_4^{2-}$-grown cells. The large peak of neutral lipid radioactivity observed by TLC analysis of cysteine-grown cell lipid extracts co-chromatographed with tripalmitin. Possibly, cysteine-grown cells synthesize large amounts of triglyceride as a means of compensating for the oxidation-reduction imbalance resulting from oxygen evolution in the absence of $CO_2$ fixation.

Chlorophyll synthesis seems to be relatively unaffected by growth in cysteine. The weight ratio of total chlorophyll to total protein in cells grown on $SO_4^{2-}$ as sole sulfur source, which was measured as 0.201, was statistically significantly different from that of cells grown on cysteine as sole sulfur source, which was 0.152 ($P < 0.005$ in the $t$ test). However, the ratio of chlorophyll a to chlorophyll b, which was measured as 2.61 in $SO_4^{2-}$-grown cells and 2.80 in cysteine-grown cells, was not significantly different under the two growth conditions ($P > 0.2$ in the $t$ test). Certainly the effect on chlorophyll synthesis was not commensurate with that on sulfolipid synthesis.

**Electron microscopy of *Chlorella* grown on cysteine as sulfur source.** Electron micrographs of *Chlorella* grown on cysteine or $SO_4^{2-}$

TABLE 2. *Conversion of $^{35}S$-labeled precursors to sulfolipid*

| Label | Wt of lipid obtained (mg) | Total counts in lipid (dpm) | Total counts in sulfolipid | | nmol of sulfolipid/mg of lipid | |
|---|---|---|---|---|---|---|
| | | | A | B | A | B |
| [$^{35}S$]cysteine[a] (200 $\mu$Ci/100 $\mu$mol) | 3.0 | 17,500 | 2,231 | 5,253 | 0.34 | 0.80 |
| $^{35}SO_4^{2-}$ (200 $\mu$Ci/100 $\mu$mol) | 5.2 | 1,142,000 | 141,656 | 1,002,284 | 17 | 227 |

[a] Cysteine is sole sulfur source.

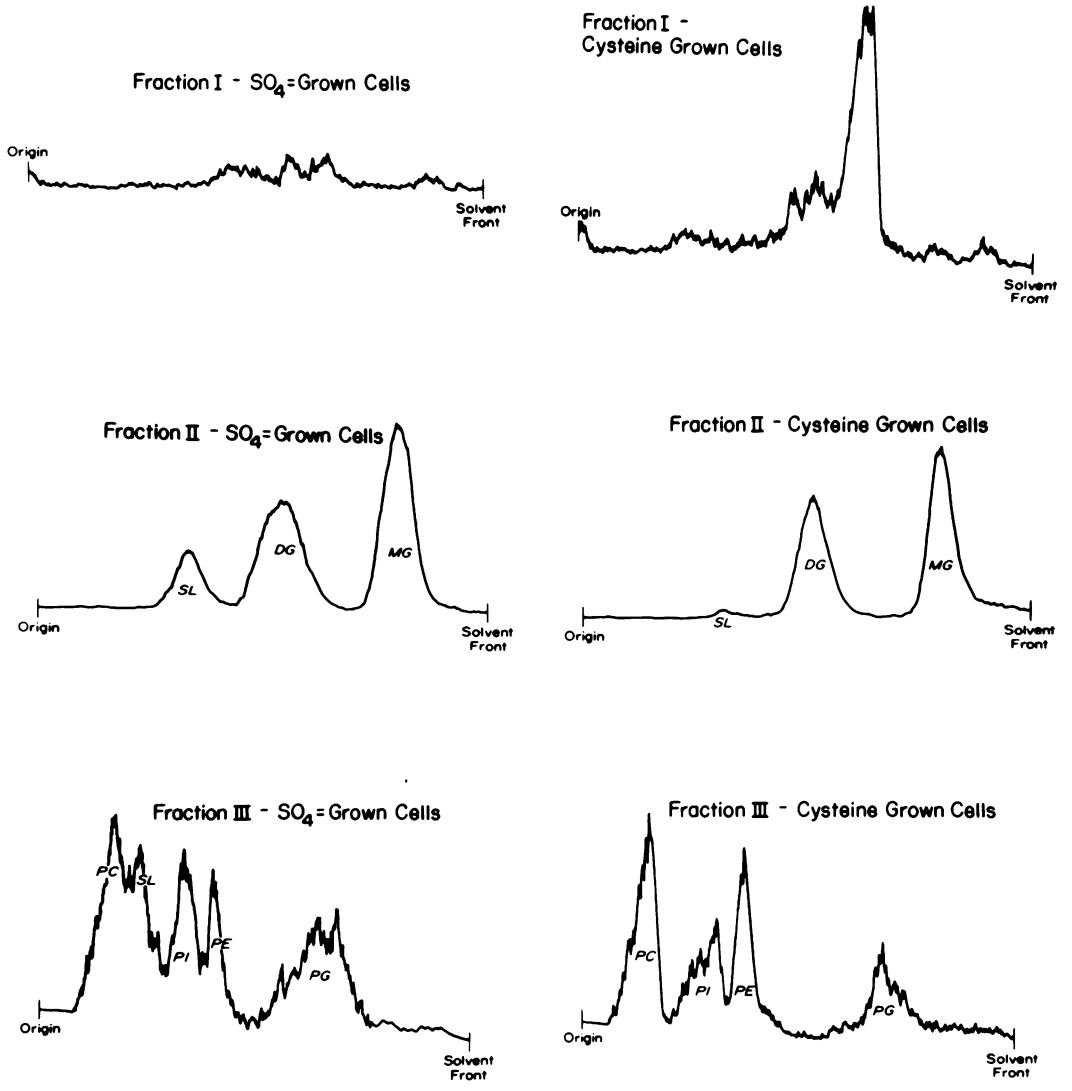


FIG. 1. *Radio-TLC analysis of lipid fractions obtained by silicic acid column chromatography. Fraction I contains neutral lipids, fraction II contains glycolipids, and fraction III contains phospholipids. Lipids were labeled by growing cells on medium supplemented with [$^{14}$C]sodium acetate. Abbreviations: SL, sulfolipid; DG, digalactosyl diglyceride; MG, monogalactosyl diglyceride; PC, phosphatidyl choline; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol.*

TABLE 3. *In vivo studies of $CO_2$ fixation*

| Sulfur Source | Glucose? | Rate of $CO_2$ fixation (nmol/h per μg of chlorophyll | Rate of $O_2$ evolution (nmol/h per μg of chlorophyll |
|---|---|---|---|
| $SO_4^{2-}$ | No | 41.4 | 75 |
| $SO_4^{2-}$ | Yes | 8.5 | 54 |
| Cysteine | Yes | 2.7 | 62 |
| $SO_4^{2-}$ + cysteine | Yes | 8.5 | |

as sole sulfur source are shown in Fig. 2 and 3. These electron micrographs show that the thylakoid structure in cysteine-grown cells exists as a stacked membrane. Higher-magnification photographs (not shown) revealed that chloroplast ribosomes were present in cysteine-grown cells. Some algal mutants defective in photosynthesis have been shown to be defective in thylakoid stacking (15) or in the synthesis of chloroplast ribosomes (16).

A striking feature of the cysteine-grown cells

TABLE 4. *In vitro studies of $CO_2$ fixation*[a]

| Reaction[b] | Sulfur source | Activity (nmol/h per mg of protein) |
|---|---|---|
| R5P → PGA | Cysteine | 29 |
| | $SO_4^{2-}$ | 270 |
| R5P → Ru5P | Cysteine | 49,700 |
| | $SO_4^{2-}$ | 49,500 |
| RU5P → Ru1,5diP | Cysteine | 370 |
| | $SO_4^{2-}$ | 1,850 |
| Ru1,5diP → PGA | Cysteine | 260 |
| | $SO_4^{2-}$ | 260 |

[a] All cultures were supplemented with 0.5% glucose and grown in the light.

[b] Abbreviations: R5P, ribose-5-phosphate; PGA, 3-phosphoglyceric acid; Ru5P, ribulose-5-phosphate; Ru1,5diP, ribulose-1,5-diphosphate.

is the large, lightly stained inclusions that seem to have displaced other cellular organelles to the periphery of the cell. Under the conditions of staining (osmium fixation), these inclusions are probably lipid droplets, which is consistent with the previous demonstration of large quantities of triglyceride in these cells.

## DISCUSSION

An important motivation for finding conditions under which a cellular component is not biosynthesized is that the resultant pathology of the cell under these conditions indicates the function of that component. Although the plant sulfolipid was first reported by Benson et al. in 1959 (7), little has been done since that time to elucidate its function. The physical-chemical properties of the sulfolipid have led to two reasonable hypotheses concerning its function. On the basis of the interaction of sulfolipid with chlorophyll in monolayers, Trosper and Sauer (35) suggested that the sulfolipid may play a role in orienting chlorophyll in the lipid bilayer of the chloroplast. On the other hand, Haines (18) has suggested that the low pK of the sulfolipid would make it an excellent ion carrier. Clearly, the deficit in $CO_2$ fixation apparently due to a lack of phosphoribulokinase activity in sulfolipid-deficient cells suggests a rather different interpretation for the biological role of sulfolipids.

It is interesting to speculate that there is a relationship between phosphoribulokinase and the sulfolipid. Since no requirement for this lipid is demonstrable for activity of purified phosphoribulokinase preparations (30), an attractive alternative hypothesis is that the sulfolipid represents a chloroplast binding site for the kinase.

Although the Calvin cycle enzymes are routinely assayed in supernatants (26) of cell extracts centrifuged at 138,000 × *g* for 90 min, there is evidence (21) that in the native cell these enzymes are membrane bound. In our hands about 30% of the phosphoribulokinase and ribulose diphosphate carboxylase activity is usually found in the 138,000 × *g* pellet, which tends to support this view.

If phosphoribulokinase is indeed membrane bound in native *Chlorella*, the ease with which it is dissociated (i.e., by mechanical disruption of the cell) suggests that it is an extrinsic membrane protein. Model studies with cytochrome *c* (34, 37, 38) have led to the view that extrinsic membrane proteins are held to membranes primarily by ionic interactions. Since the sulfolipid can engage in such interactions whereas the other chloroplast galactosyl glycerides cannot, it could be that this compound is particularly adapted for the binding of extrinsic proteins.

The deficit in phosphoribulokinase activity takes on added interest because it appears to be specific. Growth of cells on cysteine appears to have no commensurate effect on chloroplast lipid synthesis, pigment synthesis, and electron transport or on the other enzymes of the Calvin cycle. These findings are thus consistent with specific polar lipid-protein interactions, not random ones.

This report also has interesting implications with regard to membrane assembly. The evidence presented suggests that synthesis of membrane lipid components can be independent of one another. Thus membrane assembly does not involve a recognition of the stage of membrane completion on the part of biosynthetic enzymes. That membrane assembly may not be regulated by the membrane itself is clearly relevant to recent studies which demon-

TABLE 5. *Fractionation of lipid from cells grown on $SO_4^{2-}$ or cysteine as sole sulfur source on silicic acid columns*

| Fraction | Sulfur source | | | |
|---|---|---|---|---|
| | $SO_4^{2-}$ | | Cysteine | |
| | Counts | % Total counts | Counts | % Total counts |
| I–Neutral lipids | 15,815 | 4.6 | 50,918 | 17.5 |
| II–Glycolipid and sulfur lipid | 94,428 | 27 | 51,823 | 18 |
| III–Polar lipid | 78,910 | 23 | 84,500 | 29 |
| Noneluted counts | 154,583 | 45.4 | 103,692 | 35.5 |

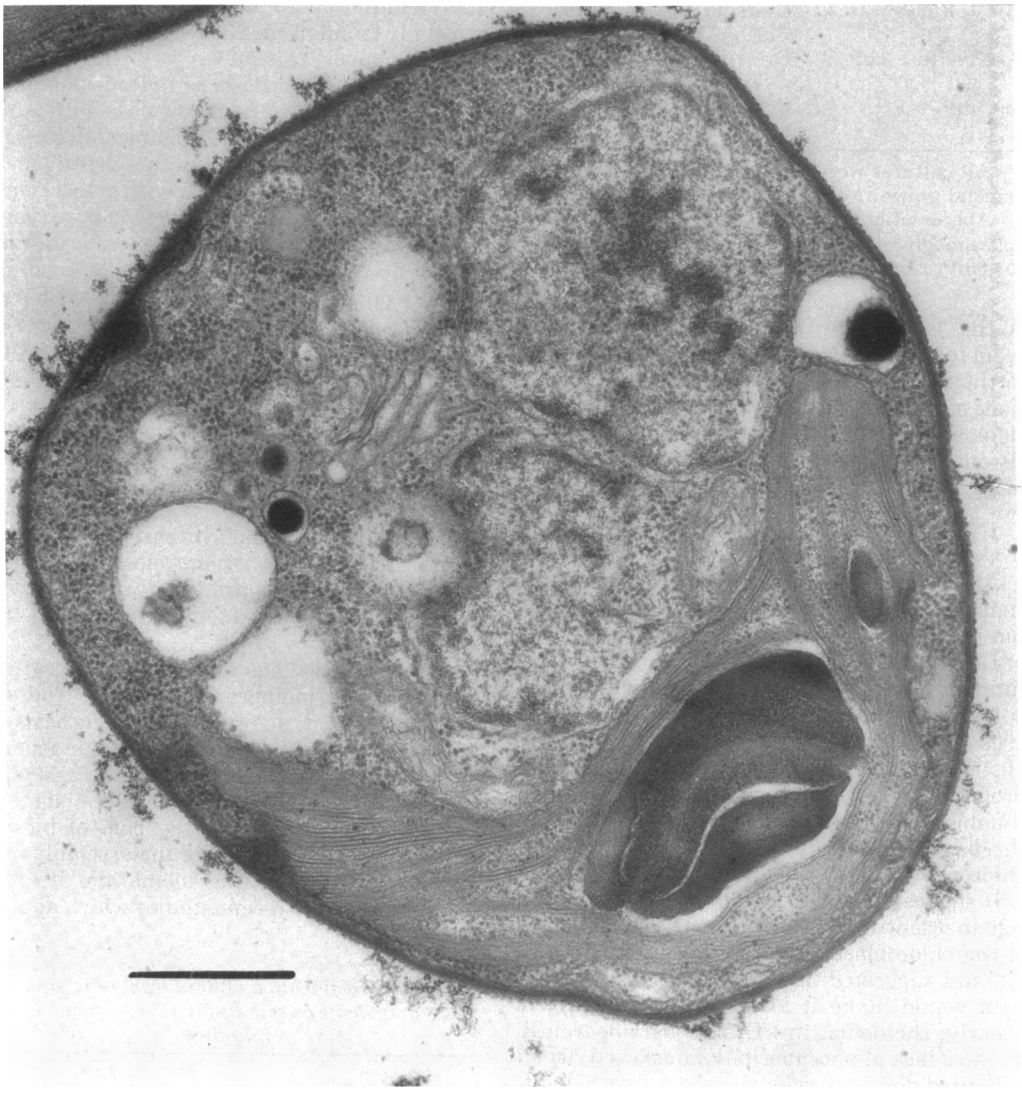

FIG. 2. *Chlorella pyrenoidosa grown on $SO_4^{2-}$ as sole sulfur source. Bar is 1 μm. ×30,000.*

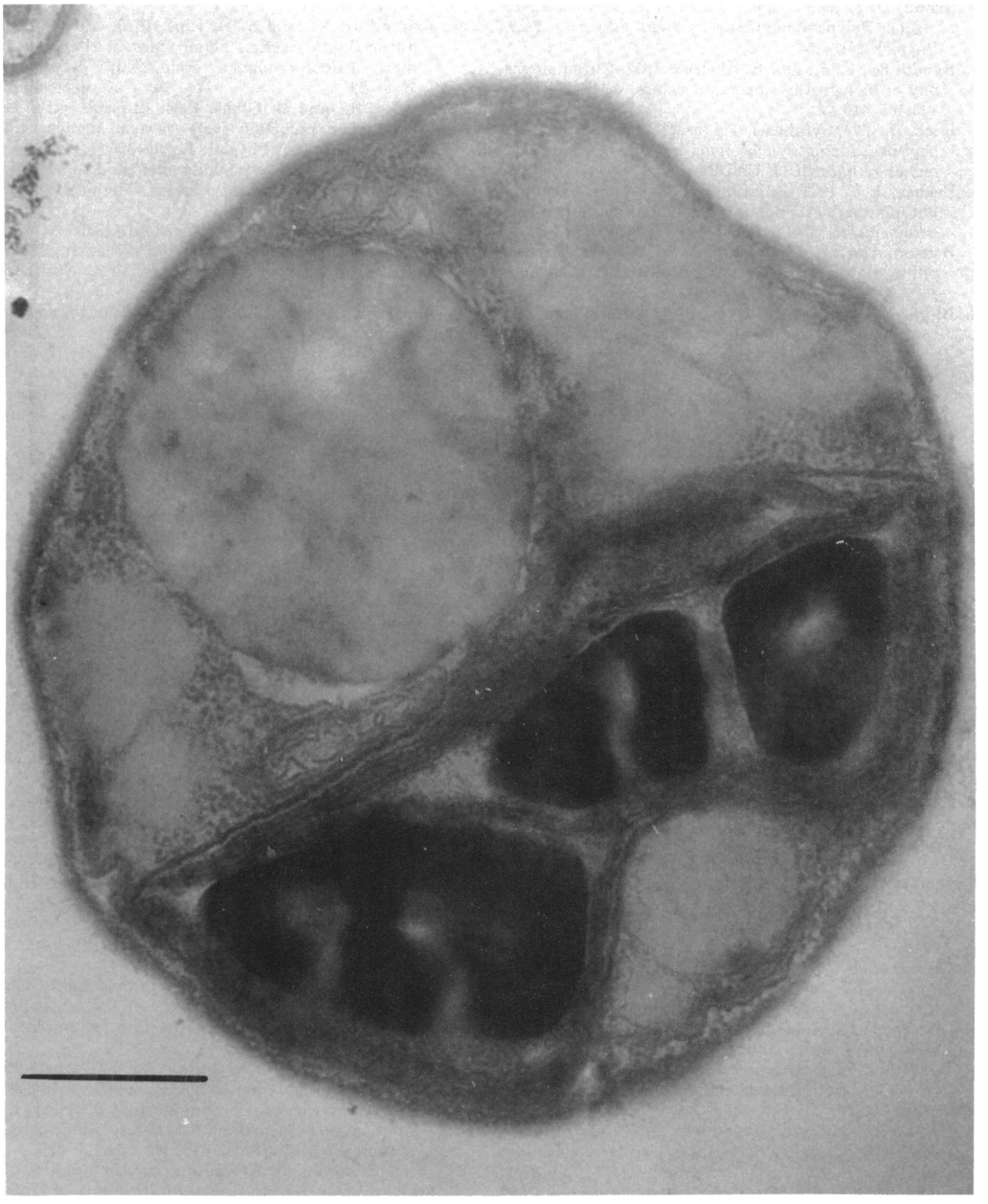

FIG. 3. *Chlorella pyrenoidosa grown on cysteine as sole sulfur source. Bar is 1 μm. ×30,000.*

strate that chloroplast lipids are assembled into membranes sequentially (5, 15).


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