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Immunocytochemical Localization of Ribulose-1,5-Bisphosphate Carboxylase in the Pyrenoid and Thylakoid Region of the Chloroplast of *Chlamydomonas reinhardtii*[1]

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ABSTRACT

The distribution of the large and small subunits of ribulose-1,5-bisphosphate carboxylase in the chloroplast of *Chlamydomonas reinhardtii* was studied by immunoelectron microscopy by labeling Lowicryl-embedded sections with antibody to each subunit followed by protein A-gold. In light-harvested synchronously dividing cells, antibodies to each subunit heavily labeled the pyrenoid, whereas the thylakoid region of the plastid was lightly labeled. By estimating the volume of each chloroplast compartment, it was determined that approximately 40% of the total small subunit in the plastid and 30% of the large subunit are localized in the thylakoid region, presumably in the stroma. In synchronously dividing cells exposed to an extended dark period, the amount of labeling of the pyrenoid region by antibody to the small subunit stayed constant, but the labeling of the thylakoid region decreased. In stationary phase cells, the proportion of the label over the pyrenoid is higher than in synchronously dividing cells suggesting that the pyrenoid may be a storage organelle.

Pyrenoids are distinctive structures found in the chloroplasts of many species of algae from all algal classes and also in the plastid of the bryophyte *Anthoceros*. They are recognized as dense, homogeneous, occasionally crystalline, regions of the chloroplast stroma from which plastid ribosomes and DNA are excluded. Thylakoids are either absent in the pyrenoid region or reduced to varying degrees. The pyrenoids of green algae and *Anthoceros* are especially conspicuous for they are demarcated from the rest of the chloroplast by a shell of starch grains. In the groups of algae which store starch or a related polysaccharide outside the chloroplast, this photosynthate frequently caps the pyrenoid region of the chloroplast.

Although it was realized early that pyrenoids were proteinaceous bodies, it was not until 1971 that Holdsworth (13) showed that pyrenoids isolated from the green alga *Eremosphaera viridis* had high specific RuBPCase[3] activity. Two polypeptides of the same size as the two subunits of RuBPCase made up 90% of the total pyrenoid protein. Similar results were subsequently obtained for pyrenoids isolated from the prasinophycean alga *Micromonas squamata* (24) and the brown alga *Pilayella littoralis* (14). In addition, the 56 and 14 kD proteins from the isolated pyrenoids of *P. littoralis* give similar tryptic peptide maps and amino acid profiles as purified whole cell RuBPCase (15). Similarly, the 52 kD polypeptide of pyrenoids isolated from the green alga *Bryopsis maxima* and the large subunit of RuBPCase yield similar peptide maps (25). Vladimirova *et al.* (31) studied the distribution of RuBPCase in the chloroplasts of two green algae *Chlamydomonas reinhardtii* and *Dunaliella salina* by indirect immunofluorescence. They observed an intense fluorescence over the pyrenoid. Vladimirova and coworkers have also shown that the size of the pyrenoid in *Chlorella* correlates with the amount of RuBPCase per cell (papers cited in Ref. 31). Likewise, Goodenough and Levine (8) showed that the ac-20 mutant of *C. reinhardtii* which lacks RuBPCase has no or rudimentary pyrenoids.

These studies clearly demonstrate that pyrenoids are rich in RuBPCase, but whether this RuBPCase is functional in the pyrenoid or is simply stored there is not known. Although other polypeptides have been observed in isolated pyrenoids, no other enzyme of the reductive pentose phosphate cycle has been found there (26). Nitrase reductase, however, has recently been found in the pyrenoids of several green algae (17, 18). Nor has it been definitely established whether RuBPCase is also present throughout the chloroplast stroma as it is in non-pyrenoid containing algae and higher plants (10, 16, 22).

In this study we confirm by immunoelectron microscopy that RuBPCase is concentrated in the pyrenoid of *C. reinhardtii*. In synchronously dividing cells, however, 30 to 40% of the total enzyme is estimated to be present in the thylakoid region of the chloroplast. The effects of darkness and of cessation of cell growth on the distribution of the enzyme are also reported.

MATERIALS AND METHODS

Cell Culture. *Chlamydomonas reinhardtii* Dangeard 137c$^+$ was obtained from the Culture Centre of Algae and Protozoa (Cambridge, UK; No. 11/32a). Cells were grown either synchronously at 25°C in an acetate-containing medium (21) on an alternating 12 h light: 12 h dark cycle or in continuous light at 20°C in Beijerinck's medium (30). The light intensity in each case was 4300 lux. The synchronously dividing cells were harvested at h 1.5 of the light period at a cell density of 2.9×10^6 cells/ml. Cells exposed to an extended dark period were placed in the dark at h 8 of the previous light cycle and harvested after 17.5 h of continuous darkness. This culture also contained 2.9×10^6 cells/ml. The continuous light cultures were harvested for EM when the cells were in the late stationary phase of growth.

Fixation and Embedding. Cells were fixed in 1% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 90 min at 4°C, rinsed in buffer, and blocked in 2% (w/v) agar. The agar blocks

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[3] Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); IgG, immunoglobulin G; SSU, small subunit of RuBPCase; LSU, large subunit.

were dehydrated in a graded ethanol series and embedded in Lowicryl K4M (J.B. EM Services, Montreal) at −18°C as described previously (16).

Immunostaining. Antibodies to the small and large subunit of RuBPCase were raised in rabbits against SDS-dissociated subunits of *C. reinhardtii* holoenzyme (6), and were kindly given to us by N.-H. Chua (Rockefeller University, New York). Colloidal gold was prepared according to the method of Frens (7) and coupled to protein A (Pharmacia, Dorval, Quebec) by the method of Roth *et al.* (23). Pale gold sections were cut and mounted on carbon-stabilized Formvar-coated nickel grids. Immunostaining was performed by placing the grids section-side down on drops of the following solutions: PBS, 5 min; 0.5% or 1% (w/v) ovalbumin, 15 min; antibody at 50 to 500 μg of IgG per ml, 60 min; rinse in jets of PBS; protein A-gold diluted 1:10, 30 min. All dilutions were in PBS. Sections were subsequently stained with uranyl acetate and lead citrate and viewed in a Philips EM 410 at 80 kV. In control experiments, antibody was replaced with rabbit nonimmune IgG (Sigma), or the antibody was omitted altogether and sections were incubated in PBS alone prior to protein A-gold labeling.

Quantitative Evaluation. The density of labeling of each cell compartment was obtained by determining the number of gold particles per μm^2 of compartment sectioned. Area determinations and gold particle counts were made using a Zeiss MOP-3.

The volume of the pyrenoid and the thylakoid region of the chloroplast of the light-harvested synchronously dividing cells was determined by measuring the length and width of the pyrenoid and of the surrounding starch shell in 36 micrographs in which the pyrenoid was cut in medial or near medial section. The volume of the pyrenoid and of the starch shell plus pyrenoid compartment was determined from the formula of an oblate spheroid. Cell volume was determined similarly employing the formula for a prolate spheroid. Chloroplast volume was estimated to be 40% of the cell volume. The chloroplast of *C. reinhardtii* has been shown by serial section EM to occupy 40% of the cell volume in mating type + gametes (28) and in synchronously dividing log phase cells grown under a 14 h light: 10 h dark regime and harvested at the beginning of the light period (3).

RESULTS

Figure 1 shows the labeling by anti-SSU of a synchronously dividing cell of *C. reinhardtii* harvested at h 1.5 in the light. The pyrenoid region of the chloroplast is heavily labeled. The thylakoid region of the chloroplast is lightly labeled, whereas the mitochondria and the rest of the cytoplasm are unlabeled. The outer edge of the cell wall is marked by a nonspecifically bound layer of gold particles.

Figure 2 shows a cell from the same culture labeled with anti-LSU. The pyrenoid is moderately heavily labeled whereas the thylakoid region of the chloroplast is very lightly labeled. The starch grains of the plastid, the nucleus, and the cytoplasm of the cell are unlabeled.

Table I compares the density of labeling of the various chloroplast compartments with that of the remainder of the cell or with the nucleus. The nucleus being a dense structure is always slightly more heavily labeled than the cytoplasm, so in those cases where it was impossible to make an accurate count of the total cytoplasmic gold particles, the nuclear labeling is used as the background control. With anti-SSU, the labeling of the thylakoid region of the chloroplast is significantly above that of the nucleus ($P < 0.001$). With anti-LSU, although there is less labeling of the thylakoid region, the labeling is still significantly higher than that over the remainder of the cell ($P < 0.01$).

The effect of light on the labeling of the pyrenoid and the thylakoid region of the chloroplast by anti-SSU was determined by exposing cells to an extended dark period. This dark period commenced at h 8 of the previous 12 h light period and extended for 17.5 h. This experiment used the same concentration of anti-SSU (500 μg/ml) and the same protein A-gold preparation so the labeling densities can be directly compared. The extended dark period had no effect on pyrenoid labeling ($P > 0.1$), but the labeling of the thylakoid region was significantly decreased ($P < 0.05$), although it remained significantly above background ($P < 0.001$).

Table I also gives the density of labeling by anti-SSU and anti-LSU of different chloroplast compartments in stationary phase cells which had been grown in continous light. Although a similar pattern of labeling was observed in these cells, the labeling of the thylakoid region by anti-LSU was not significantly above that observed over the rest of the cell ($P > 0.05$). With anti-SSU, the thylakoid region ($P < 0.01$) as well as the pyrenoid were significantly labeled.

As controls, antibody was replaced by nonimmune IgG or the antibody binding step was replaced by incubation in PBS prior to treatment with protein A-gold. No labeling was observed with either treatment other than a slight tendency of protein A-gold alone to stick to the dense pyrenoid (Table I).

The pattern of labeling observed in Figures 1 and 2 gives the visual impression that almost all the RuBPCase in the plastid is localized in the pyrenoid region. This is a misleading impression caused by the fact that one is observing the labeling of antigens at the surface of a two-dimensional section. Since the volume of the pyrenoid is very small compared with that of the thylakoid region of the chloroplast, even a low level of genuine labeling of the thylakoid region indicates that a significant amount of enzyme is present in this compartment. In order to determine what proportion of the total plastid enzyme is localized in the pyrenoid, we determined the mean volume of the pyrenoid (2.4 μm^3) and of the thylakoid region of the chloroplast (35.6 μm^3) in the light-harvested synchronously dividing cells and calculated the percentage of SSU and LSU present in each compartment. Approximately 40% of the total SSU in the chloroplast is localized in the thylakoid region and 60% in the pyrenoid. Of the total LSU in the plastid, 30% is in the thylakoid region and 70% in the pyrenoid.

DISCUSSION

This study confirms that RuBPCase is heavily concentrated in the pyrenoid region of the chloroplast of *C. reinhardtii*. Although the thylakoid region was only lightly labeled, we estimate that in synchronously dividing cells, 40% of the total SSU in the plastid and 30% of the LSU is localized in the thylakoid region, presumably in the stroma. In several previous studies on isolated pyrenoids, RuBPCase was also found in the high speed supernatant fraction from lysed whole cells (13, 24) or in the soluble chloroplast fraction (25), indicating that RuBPCase is also present in the chloroplast stroma outside the pyrenoid. However, in these biochemical studies it was not possible to be certain that the RuBPCase of the pyrenoid had not been solubilized during isolation. Vladimirova *et al.* (31) reported that the entire chloroplast of *C. reinhardtii* is faintly fluorescent when immunolabeled by anti-RuBPCase and concluded that a very small amount of RuBPCase is present in the thylakoid region of the chloroplast. Immunocytochemistry at the electron microscope level has allowed us to show conclusively that the thylakoid region of the chloroplast of dividing cells of *C. reinhardtii* contains RuBPCase. Furthermore, we show that the observed low level of labeling of the thylakoid region in fact represents a large fraction of the total plastid RuBPCase since the volume of the thylakoid region is 15 times greater than that of the pyrenoid.

In Table I, it is not possible to compare directly the density of labeling of each compartment by anti-LSU and anti-SSU because

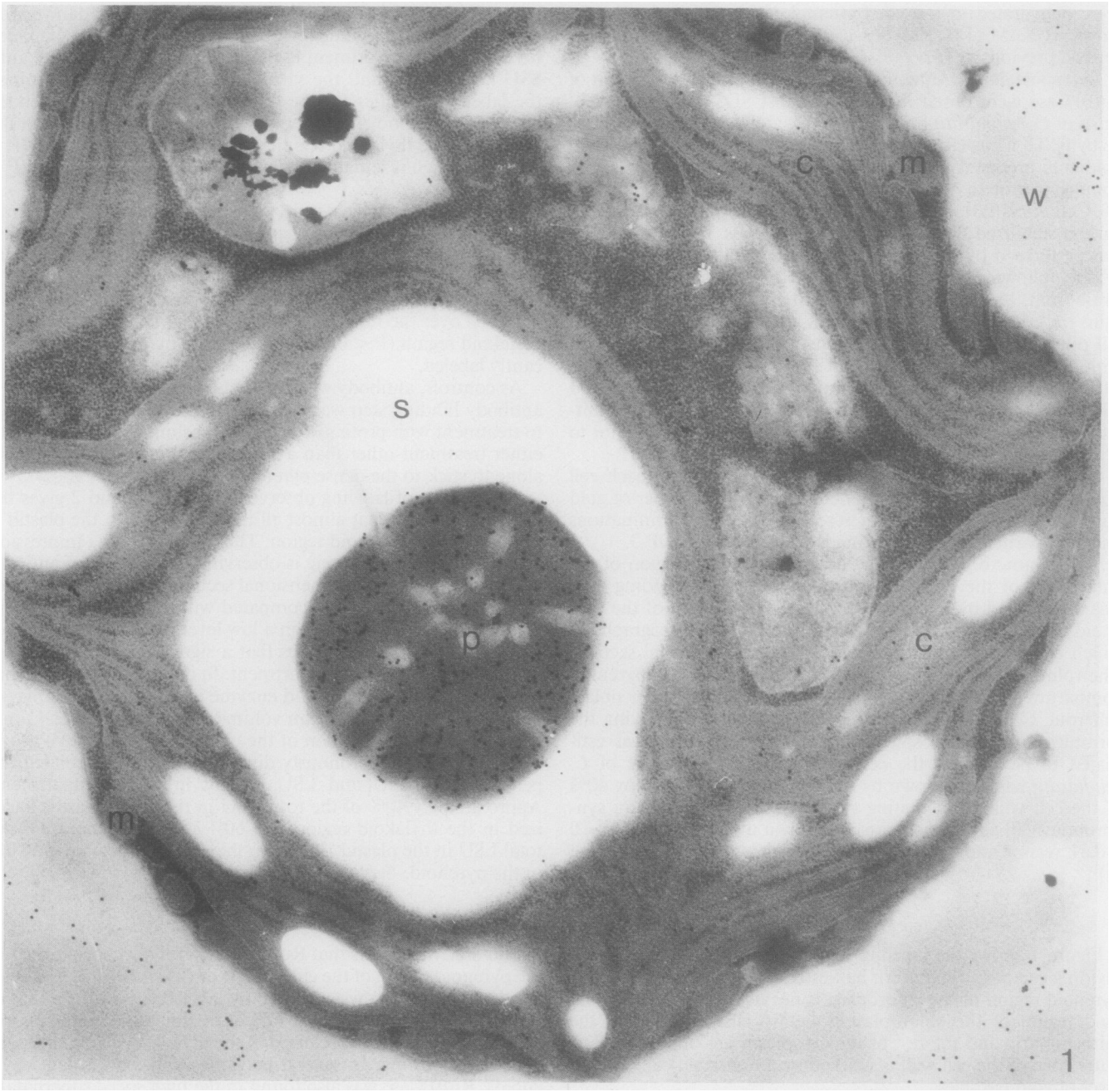

FIG. 1. Synchronously dividing cell labeled with anti-SSU. Gold particles are heavily concentrated over the pyrenoid (p), largely absent from the starch shell (s) and stroma starch grains. The thylakoid region of the chloroplast (c) is lightly labeled. The mitochondria (m) and the cell's cytoplasm are unlabeled. A layer of nonspecifically bound gold particles marks the outer boundary of the cell wall (w) (×33,800).

equal amounts of the two antibodies will not necessarily react with the same amount of antigen. Also with this technique, the absolute density of labeling in a given experiment is dependent on the particular preparation of protein A-gold used. In Table I, the only two samples which were labeled by the same protein A-gold preparation are the anti-SSU-labeled light-harvested and dark-treated synchronously dividing cells. Although the labeling by anti-SSU and anti-LSU cannot be directly compared, different patterns of labeling are significant. We always observed proportionally more labeling of the thylakoid region by anti-SSU than by anti-LSU. This may indicate that there is a pool of excess free SSU in the thylakoid region. Primary leaves of pearl millet contain more SSU than LSU (2), but in *C. reinhardtii* excess SSU has been shown to be degraded (27, 29). Another possibility to explain the apparent discrepancy in the amounts of SSU and LSU in the thylakoid region is that the newly synthesized LSU is associated with the recently characterized LSU binding protein (12) and this binding protein masks antibody-binding sites. Barraclough and Ellis (1) found that newly synthesized LSU was not precipitated by anti-RuBPCase which did precipitate LSU prepared from holoenzyme.

The function of pyrenoids remains obscure. It is not known whether the pyrenoid stores excess RuBPCase or whether the RuBPCase in the pyrenoid actively fixes CO_2. The fact that in late stationary phase cells, proportionally more of each subunit is in the pyrenoid (Table I) suggests that the pyrenoid may be a storage organelle. The observation that after an extended dark period, the amount of SSU decreased in the thylakoid region but

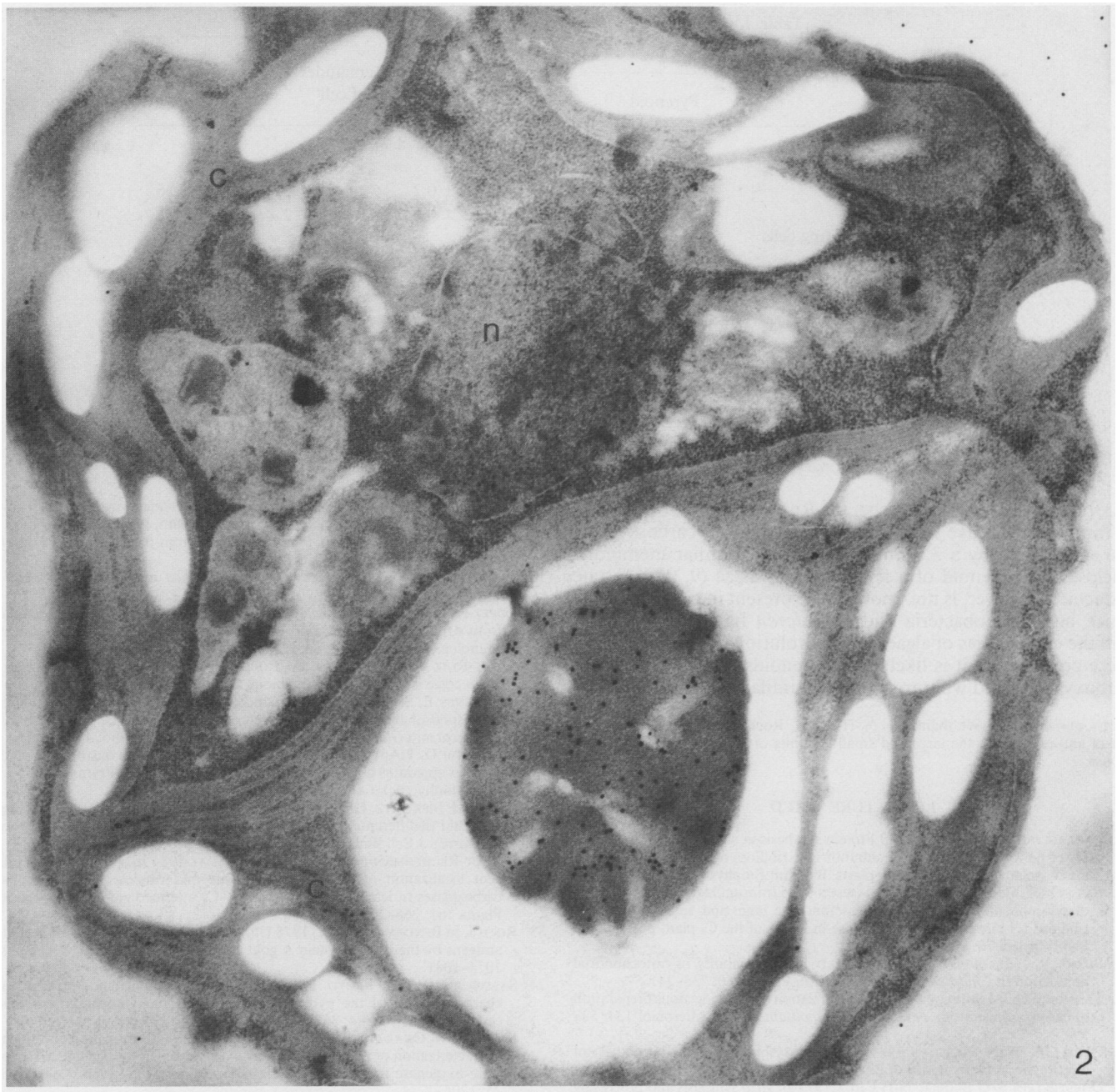

FIG. 2. Synchronously dividing cell labeled with anti-LSU. The pyrenoid is moderately heavily labeled whereas the thylakoid region of the chloroplast (c) is very lightly labeled. The starch shell surrounding the pyrenoid and the stroma starch grains are unlabeled as is the nucleus (n) and cytoplasm of the cell (×33,800).

not in the pyrenoid could also be used as evidence that the active pool of enzyme is in the thylakoid region. At present it is not known if pyrenoids contain any other enzymes of the reductive pentose phosphate cycle. Holdsworth (13) originally suggested that ribose-5-phosphate isomerase and ribulose-5-phosphate kinase were present in the pyrenoid of *Eremosphaera*. However, Satoh *et al.* (26) have more recently demonstrated by peptide mapping that a minor 42 kD protein found in the pyrenoid of another green alga, *Bryopsis maxima*, is not the 41 kD ribulose-5-phosphate kinase of the cell. The recent unexpected discovery of nitrate reductase in the pyrenoids of several green algae (17, 18) suggests that this enzyme might function there. However, the authors observed that the thylakoid region of the chloroplast of *C. reinhardtii* was also moderately labeled (18), but concluded that only a small percentage of the enzyme was localized there. However, when one considers the small volume of the pyrenoid relative to the rest of the chloroplast, one realizes that a large percentage of the nitrate reductase is localized in the thylakoid region of the plastid. Again one does not know if the enzyme is functional in each compartment. Another often suggested function of the pyrenoid of green algae is that they are involved in starch synthesis (9). Clearly immunocytochemical studies should be done to determine if the enzymes of starch synthesis are located in the pyrenoid.

Pyrenoids are in many ways similar to the carboxysomes found in autotrophic prokaryotes and in cyanelles. RuBPCase is the major component of both (5, 19). As in pyrenoids, other enzymes of the reductive pentose phosphate cycle appear to be absent (4,

Table I. *Density of Labeling Over Various Cell Compartments*

	Chloroplast			Remainder of cell	Nucleus	n^a
	Pyrenoid	Starch shell	Thylakoid region			
	gold particles/μm^2 ± SEM					
Synchronously dividing cells						
Anti-SSU	132.6 ± 2.5	2.7 ± 0.3	12.9 ± 0.7		7.0 ± 0.6[b]	26
Anti-LSU	76.9 ± 4.8	1.2 ± 0.3	4.1 ± 0.6	2.0 ± 0.3	2.6 ± 0.8[c]	15
Synchronously dividing cells (17.5 h dark)						
Anti-SSU	125.4 ± 4.6	3.2 ± 0.5	10.9 ± 0.5		5.5 ± 0.6[b]	25
Stationary phase cells						
Anti-SSU	185.1 ± 23.0	2.8 ± 0.9	13.3 ± 2.2	5.6 ± 0.8		15
Anti-LSU	150.5 ± 11.8	1.4 ± 0.3	4.9 ± 0.6	3.3 ± 1.2		14
Controls[d]						
Nonimmune IgG	1.3 ± 0.4	0.3 ± 0.1	1.7 ± 0.4	1.0 ± 0.2		13
pAg alone	5.8 ± 2.7	0.6 ± 0.5	1.0 ± 0.6	0.4 ± 0.3		5

[a] Number of cell sections analyzed. [b] Mean of eight nuclei. [c] Mean of six nuclei. [d] Controls were performed on stationary phase cells.

11). It is also not known whether the RuBPCase in carboxysomes is functionally active *in vivo*. Most, but not all, carboxysomes are surrounded by a 3.5 nm membrane. A similar membrane encloses the pyrenoid of a few species of algae (9, 20). Nitrate reductase, however, is not known to be present in carboxysomes. Since both cyanobacteria and *Prochloron* have carboxysomes and the chloroplasts of algae are the evolutionary descendants of these prokaryotes, it is likely that pyrenoids have evolved from carboxysomes and will prove to have similar functions.

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