THE ISOLATION AND PARTIAL CHARACTERIZATION OF THE PYRENOID PROTEIN OF *EREMOSPHAERA VIRIDIS*

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ABSTRACT

The pyrenoids of *Eremosphaera viridis*, a green alga, were isolated by density gradient centrifugation and their physical and enzymatic properties were studied. The ultraviolet absorption spectrum of sodium dodecyl sulfate (SDS) extracts of pyrenoids showed a single peak at a wavelength of 277 nm, indicating the presence of protein and the probable absence of nucleic acid. Upon electrophoresis on polyacrylamide gels containing SDS, 16 bands were resolved of which two, together, accounted for 90% of the total protein on the gels. The molecular weights of these two proteins were estimated to be 59,000 and 12,300 and the ratio by weight of the larger to the smaller protein was found to be 2:1. The physical and enzymatic properties of these two proteins were found to closely resemble the properties reported in the literature for the subunits of fraction I protein. Both pyrenoids and fraction I protein are localized in the chloroplast, and both have two principal protein components. The molecular weights and relative ratio of the two pyrenoid components are very similar to those of the two components of fraction I protein. The pyrenoid was found to contain a high specific activity of ribulose-1,5-diphosphate carboxylase which is the same enzymatic activity exhibited by fraction I protein. The presence of ribose-5-phosphate isomerase and ribulose-5-phosphate kinase activities was also noted in pyrenoid preparations. It is suggested that the pyrenoid contains fraction I protein and possibly other enzymes of the Calvin-Bassham carbon dioxide fixing pathway.

INTRODUCTION

In the chloroplasts of many species of algae and of some species of liverworts are found round, angular, or irregularly shaped bodies called pyrenoids (20, 55). Most studies of the pyrenoid have concentrated on its structural aspects (see 22, 23, and 26 for reviews); however, some attempts have been made to determine the chemical composition of pyrenoids by using histochemical techniques. That protein constitutes the bulk of the pyrenoid has been deduced from its staining properties. Among the most frequently cited stains used successfully on pyrenoids are safranin of Fleming's triple stain and hematoxylin (1, 3, 4, 6, 11, 12, 28, 39, 44, 54). It should be noted, however, that pyrenoids of certain algae, in particular some of the Phaeophytes (54), Rhodophyes (20), and Chrysophytes (41), do not show a strong affinity for these stains. Brown and Arnott (9) found that the pyrenoids of *Tetracystis excentrica* stained deeply with mercuric bromphenol blue, a stain shown by Mazia et al. (43) to be specific for proteins. Several studies have shown that proteolytic enzymes used as histochemical reagents are capable of digesting the pyrenoid core (9, 17, 19).

Studies to determine the enzyme content of pyrenoids have been limited to those enzymes

which can be detected histochemically. Simon (54) found alkaline phosphatase in and around the pyrenoids of *Enteromorpha*, *Rhodochorton*, and *Pylaiella* by using the Gomori reaction. Cook, also using the Gomori reaction, reported positive results for acid phosphatase in and around pyrenoids of Closterium (13).

Perhaps the most direct approach to solving the problems of pyrenoid composition and function would be to isolate this organelle in bulk and study its properties in vitro. A preliminary account of the isolation of pyrenoids of *Zygnema* has been reported by Rosowski and Hoshaw (51). They have found isolated pyrenoids to be "firm, spherical, colorless refractive bodies" when studied with the light microscope. The inability of the isolated pyrenoids to bind propiocarmine stain, as could the *in situ* pyrenoids, led the authors to suggest a difference in the chemical state between *in situ* and isolated pyrenoids.

This paper reports the isolation and characterization of the pyrenoids of *Eremosphaera viridis*, a large (250 μ in diameter), spherical, fresh-water Chlorophyte. In the present context the term "pyrenoid" will refer to both the core substance and its surrounding starch sheath (designated as pyrenoid starch). "Stroma-starch" will be used to designate the starch of the cell which is not associated with a pyrenoid core.

MATERIALS AND METHODS

Algal Culture

Axenic cultures of *Eremosphaera viridis* strain E-4[1] were grown in a medium slightly modified from the BEV medium devised by Smith and Bold (56). The MoO_3 employed in Smith's medium was replaced by the more soluble Na_2MoO_4, and the concentrations of the two vitamins used, cyanocobalamin and thiamine hydrochloride, were increased to 0.1 mg/liter each. 5% CO_2 in compressed air was continuously bubbled through a cotton filter into the medium during culture growth giving a final pH of approximately 5.5.

Every 4–7 days during growth the cells were allowed to settle in the culture flask and the depleted medium was siphoned off and replaced with fresh medium from a 6 liter reservoir bottle. Cultures were grown under conditions of constant light of approximately 130 ft-c intensity and at a temperature of approximately 20°C.

[1] The author is greatly indebted to Dr. Richard L. Smith (Department of Botany, Eastern Illinois University, Charleston, Illinois) for supplying the stock cultures used in this study.

Pyrenoid Isolation

Cultures 5 or 6 wk old were harvested by centrifugation, yielding about 15 ml of packed cells. Empty cell walls which formed a white layer above the packed cells were discarded. The cells were rinsed twice in ice-cold 0.02 M sodium phosphate buffer at pH 7.0 and were broken in the cold by 25 strokes of a teflon pestle in a hand homogenizer. The homogenate was filtered three times through 200-mesh bolting cloth (Carolina Biological Supply Co., Elon College, N. C.) to remove cell walls and unbroken cells. The resulting suspension was centrifuged in the cold for 25 min at 175 *g*. The pellet was resuspended in buffer and centrifuged in the cold at 175 *g* for 3 min. The supernatant liquid, denoted S, contained fragments of chloroplasts and was saved. The pellet containing mostly pyrenoids and stroma-starch was resuspended, centrifuged, and decanted as before. The resulting pellet was resuspended in 0.5 ml of a CsCl-sucrose solution having a density of 1.55 g/ml (42.6% w/w CsCl, 12.4% w/w sucrose, 55.0% w/w homogenization buffer). The suspension was layered on a 5 ml continuous CsCl-sucrose gradient running from a density of 1.61 g/ml at the bottom (45.5% w/w CsCl, 13.3% w/w sucrose, 41.2% w/w buffer) to a density of 1.55 g/ml at the top. The preparation was centrifuged for 5 hr at 32,600 *g* in a Beckman Model L centrifuge by which time equilibrium of the particles in the gradient was reached. The stroma-starch formed a band at a density of approximately 1.60 g/ml while the pyrenoids formed a somewhat broader zone just above the starch. Stroma-starch and pyrenoid fractions were removed from the gradient by hypodermic needles inserted through the side of the cellulose nitrate centrifuge tubes. The material in each fraction was rinsed three times with the buffer used for homogenization.

The chloroplast fragments in the supernatant liquid, S, were subjected to rehomogenization and centrifugation to remove the pyrenoids and starch grains remaining in the chloroplast material. The chloroplast fragments were then pelleted by centrifugation at 18,400 *g* for 20 min.

A flow diagram of the over-all isolation procedure is shown in Fig. 1.

Light and Electron Microscopy

Cells of *Eremosphaera viridis* were fixed for electron microscopy for 18 hr in the cold in a solution of 3% glutaraldehyde buffered at pH 7.2 with 0.01 M sodium cacodylate followed by a 3 hr postfixation in 1% osmium tetroxide in the same buffer. After several rinses with buffer, the cells were dehydrated

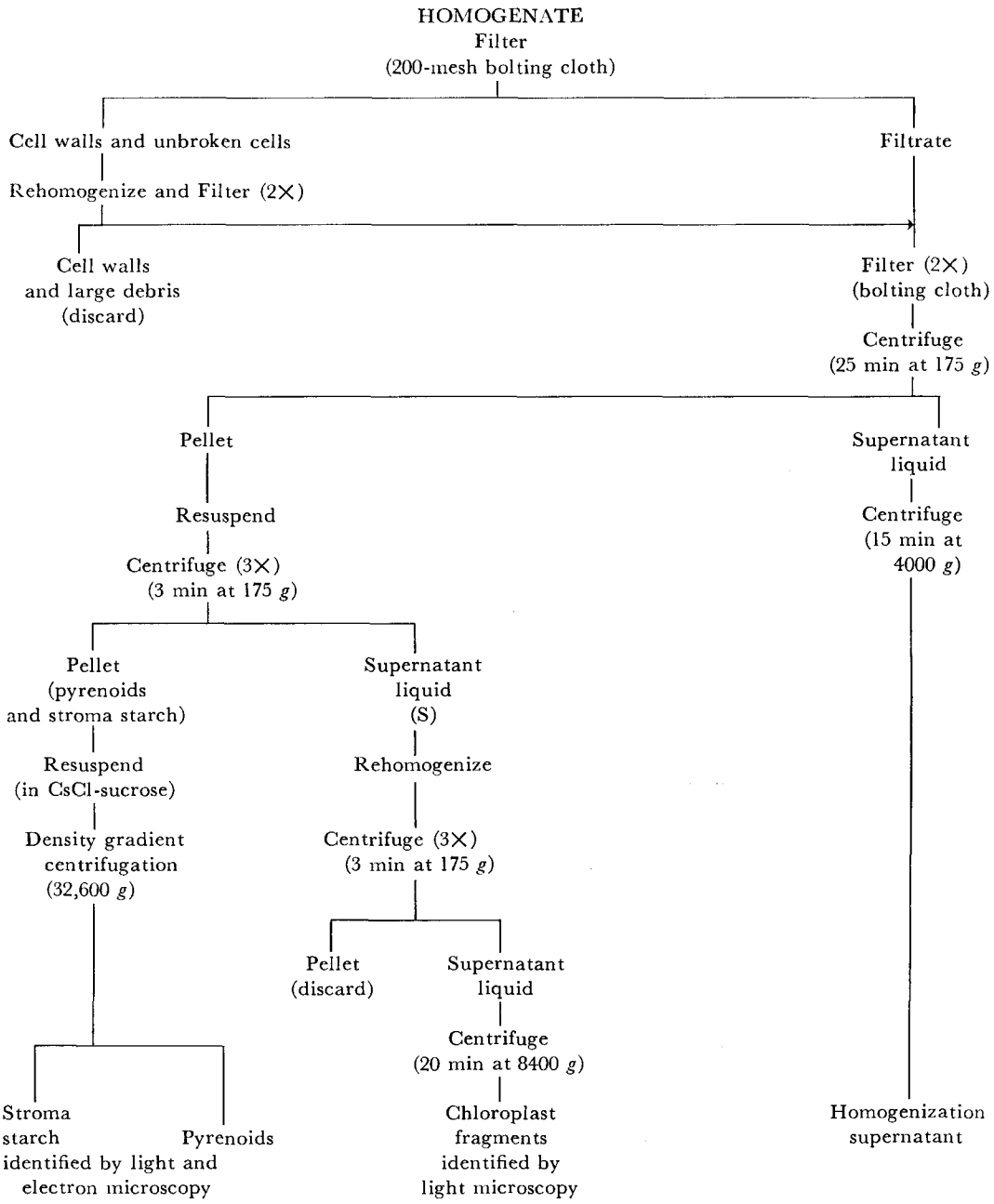


FIGURE 1 Flow diagram of procedure for isolating pyrenoids, stroma-starch, and chloroplast fragments from *Eremosphaera viridis*.

through an ethanol series and embedded in Spurr's plastic (57).

Samples of isolated pyrenoids either untreated or extracted with a 1% solution of sodium dodecyl sulfate (SDS) in pH 7.0 sodium phosphate buffer were prepared for electron microscopy by using the same procedures as were used for whole cells.

Sections cut with a duPont diamond knife (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) on a Porter-Blum model MT-2 ultramictotome were

picked up on 200-mesh copper grids coated with Formvar and carbon, and stained with uranyl acetate and lead citrate. Observations were made with an Hitachi HS-7S electron microscope at an accelerating voltage of 50 kv.

A Leitz-Ortholux photomicroscope was used for all light microscopy.

Electrophoresis

Electrophoresis was carried out on 20 cm lengths of 7.5% polyacrylamide gels containing 0.5% w/v sodium dodecyl sulfate. The samples applied to the gels contained 1% w/v SDS, 1% v/v 2-mercaptoethanol, 10% v/v glycerol, 0.002% w/v bromphenol blue (as a fast-running marker), and protein in a concentration of approximately 0.5 mg/ml all mixed in 0.02 M sodium phosphate buffer at a pH of 7.0 (32). Control samples contained all but the protein. The preparations were allowed to run for 18 hr, at the end of which time the center of the bromphenol blue band was marked with a stab from a hypodermic needle. The gels were stained overnight at room temperature with Coomassie brilliant blue R-250 and destained by swirling at 37°C in 12% trichloroacetic acid over a 2- or 3-day period. Rm values, defined as the ratio of the distance traveled by a given peptide band to the distance traveled by the bromphenol blue marker, were calculated for each band on the gels. Densitometer tracings of the gels were made on a Joyce-Loebl microdensitometer and the relative amount of protein-stain complex present in the bands of a given gel were determined by measuring with a polar planimeter the areas under the curves of the densitometer tracings (25).

Molecular weights were estimated by using a technique which involved electrophoresis in gels containing SDS (16, 53, 64). A calibration curve for this technique was made by using proteins with known molecular weights, namely pepsinogen (Worthington Biochemical Corp., Freehold, N. J.), pepsin (Calbiochem Los Angeles, Calif.), chymotrypsinogen (Worthington Biochemical Corp.), bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), pyruvate kinase (Sigma). Rm values were determined for these proteins and plotted against the logarithms of the molecular weights (Fig. 2).

Determination of Starch Weight

The number of pyrenoids and starch grains in isolated material was determined by using a Petroff-Hauser counting chamber. The pyrenoid and stroma-starch fractions were then extracted with 0.5 ml of a solution of 1% SDS buffered at pH 7.0 with 0.02 M sodium phosphate. This treatment solubilized the core material leaving only the starch which was removed by centrifugation, washed with distilled water, and dried overnight on preweighed aluminum planchets at 70°C. As controls, distilled water blanks were dried under the same conditions. The weights of the starch in each fraction could then be measured directly.

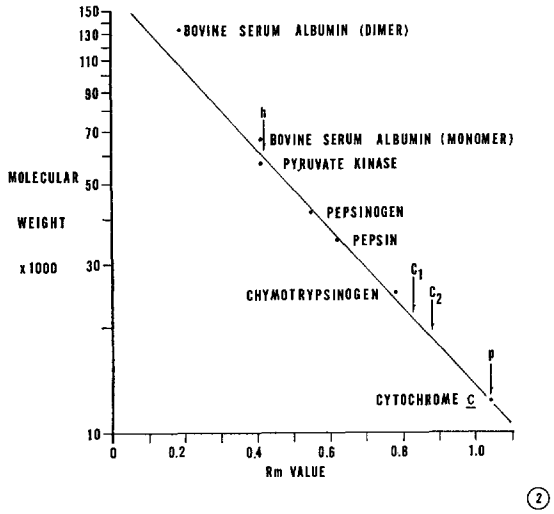


FIGURE 2 Calibration curve for determining molecular weights of proteins by electrophoresis on gels containing sodium dodecyl sulfate. Rm values are defined as the distance traveled by the protein divided by the distance traveled by a marker band of bromphenol blue. Arrows indicate Rm values of several important proteins from the pyrenoid (*h* and *p*) and chloroplast fragment (c_1 and c_2) preparations.

Estimation of Pyrenoid Size

A sample of the washed and weighed starch of each fraction was resuspended in distilled water and photographed at 1000 diameters magnification with a light microscope. Projections of the negatives to a known magnification were made with a photographic enlarger and tracings of the pyrenoids were made directly from the projected images. The widths of the extracted pyrenoid cores were measured at the widest and narrowest point on each pyrenoid, and an average "diameter" was computed. The tracings of the cores and of the starch were cut out and weighed to provide an index of the relative sizes of the irregularly shaped starch in the pyrenoid and stroma-starch samples. Discs of constant diameter were cut from the tracing papers and weighed as a test of the uniformity of the paper used.

Spectroscopy

SDS extracts of pyrenoids, stroma-starch, and chloroplast fragments were centrifuged at 3000 *g* to reduce the turbidity of the solutions. Visible and ultraviolet spectra of various known dilutions of the three extracts were recorded with a Cary 14 spectrophotometer. The ratio of the absorbance at 277 nm to the absorbance at 257 nm was used to estimate the proportion of protein to nucleic acid present in the preparations (37).

Protein Assay

The protein content of the pyrenoid and stroma-starch extracts was estimated by the method of Lowry et al. as described by Layne (37). Bovine serum albumin in 1% SDS was used as a standard.

Enzyme Analysis

Cells were homogenized and pyrenoids prepared for use in enzyme assays by essentially the same technique described earlier except that all steps were carried out in 0.1 M tris-Cl buffer at pH 7.5 containing 0.06 M $MgCl_2$, 0.004 M ethylenediaminetetraacetic acid (EDTA), 0.25 M $KHCO_3$, and 0.006 M mercaptoethanol. The CsCl-sucrose centrifugation step was omitted because of its destructive effect on enzymatic activity. The pellet of pyrenoids and stroma-starch was ground by hand with a glass rod in 0.5 ml of the buffer, to free the core material from the starch. A pellet of chloroplast fragments was ground in the same manner as the pyrenoids. The pyrenoid material, chloroplast material, and a sample of the homogenization supernatant liquid were centrifuged for 15 min at 4000 *g* in a Beckman Model L centrifuge. Each of the resulting supernatant liquids was assayed for protein content as described above and assayed for ribulose diphosphate carboxylase (RudP carboxylase) activity by the method of Racker (50). Enzyme activity was measured as a function of the rate of decrease in the absorbance of the reaction mixture at 340 nm. Specific activity as determined was defined as μM CO_2 fixed/min per mg protein.

Controls for the assay consisted of omitting either the enzyme sample or the substrate from the reaction mixture. Pyrenoid protein mixed with either the chloroplast membrane extract or the "homogenization supernatant" was also tested for enzymatic activity. In a single experiment, a sample of pyrenoid protein was placed in a boiling water bath for 4 min and then tested for carboxylase activity.

The presence of ribose-5-phosphate isomerase and ribulose-5-phosphate kinase activities was determined by substituting equimolar amounts of ribose-5-phosphate for ribulose-1,5-diphosphate in the reaction mixture (49).

A sample of the enzyme preparation for each of the three sources, pyrenoids, chloroplast fragments, and homogenization supernatant, was dialyzed against a solution of 1% SDS in 0.01 M, pH 7.0 phosphate buffer for several days and run on acrylamide gels as described above.

RESULTS

Light and Electron Microscope Observations of In Situ and Isolated Pyrenoids

Each *in situ* pyrenoid and grain of stroma-starch of *Eremosphaera viridis* appears in the electron microscope to occupy its own compartment in the chloroplast. These compartments are delineated by one or more thylakoids. Stroma-starch tends to be spheroid in shape while the pyrenoid starch tends to be irregular in both size and shape, sometimes being oval or spheroid, and sometimes being very angular. Around each starch grain and pyrenoid is a less electron-opaque zone of unknown composition bounded at its outer perimeter by chloroplast thylakoids. The spheroid pyrenoid core is completely surrounded by the pyrenoid starch with the exception of from one to three channels in the starch. Through each of these channels runs a tubular thylakoid providing the pyrenoid core with its only direct communication with the rest of the chloroplast. This "single-disc" thylakoid penetrates into the core material inside the pyrenoid and appears to be interconnected with a number of chloroplast thylakoids outside the pyrenoid as described by Bowen (7). The ground substance of the core appears to be homogeneously granular in texture, there being no crystalline regions as are seen in the pyrenoids of some algae

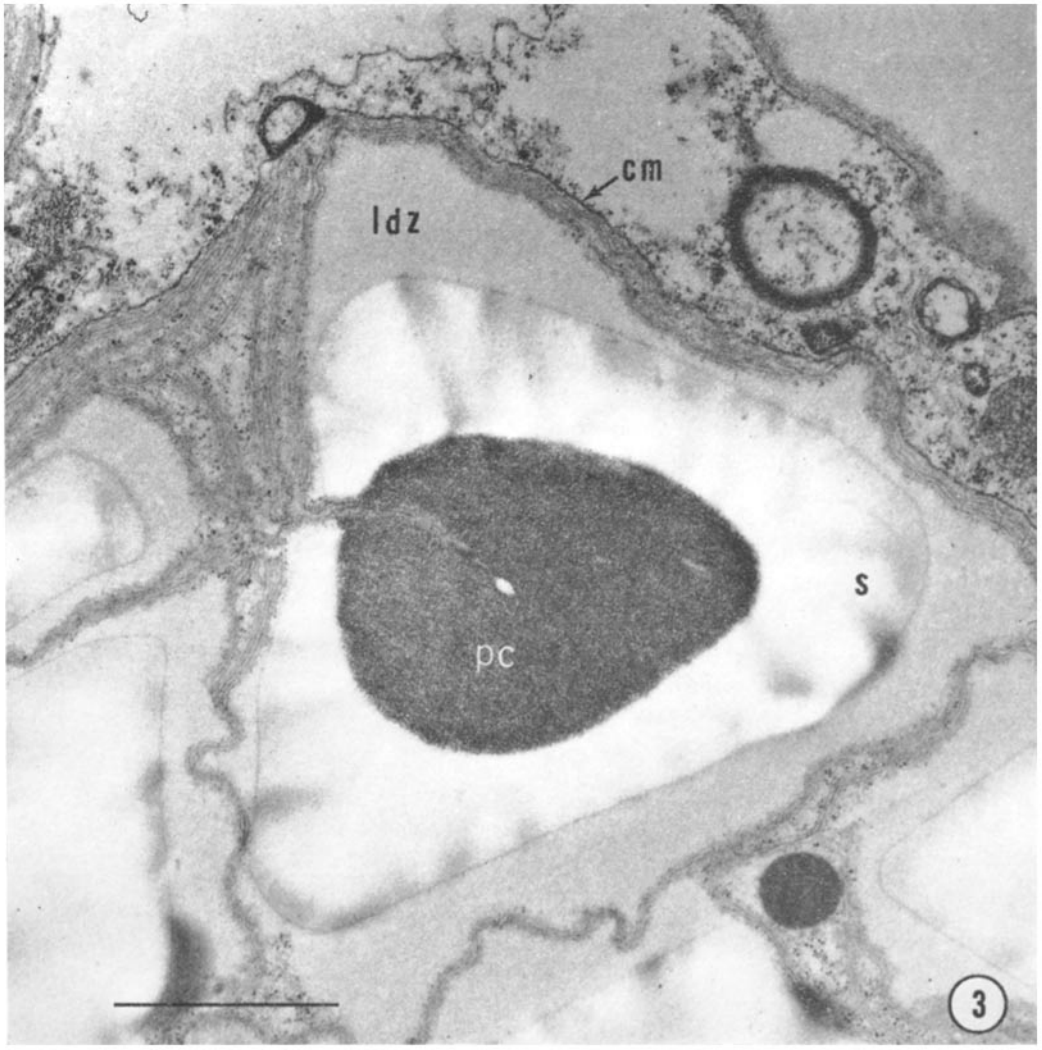


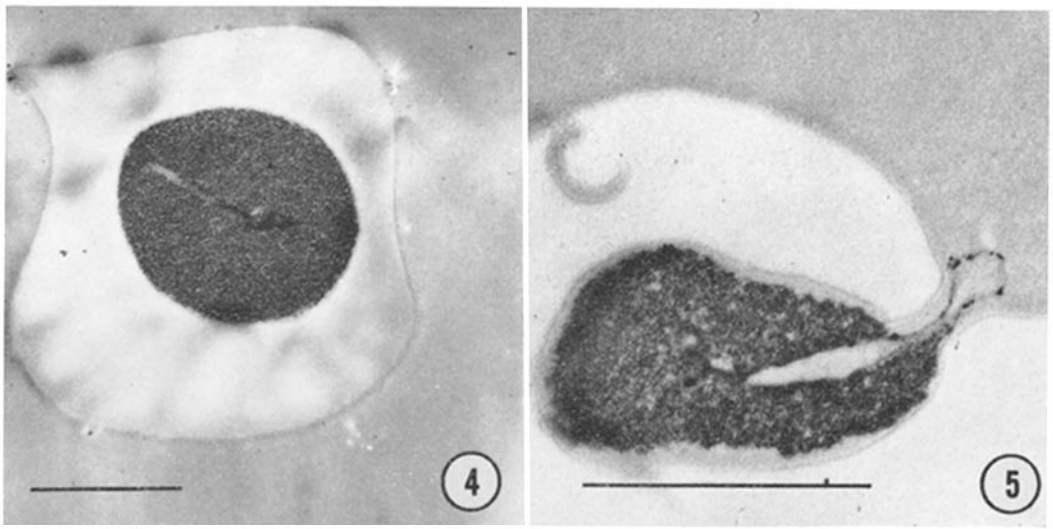
FIGURE 3 Electron micrograph of a section through an *in situ* pyrenoid of *Eremosphaera viridis*. Cells were fixed in glutaraldehyde followed by osmium tetroxide. Spurr's plastic was used for embedding. *cm*, chloroplast membrane; *ldz*, less electron-opaque zone; *pc*, pyrenoid core; *s*, starch. Bar at lower left represents 1 μ. × 30,000.

(2, 29, 35). An example of an *in situ* pyrenoid is shown in Fig. 3.

Thin sections of preparations of isolated pyrenoids viewed in the electron microscope show very little contamination except for stroma-starch. The less electron-opaque area which surrounds each starch grain *in situ* is absent here, but the cores seem to be for the most part intact (Figs. 4 and 5). The single-disc membranes which penetrate the cores were also present.

Thin sections of preparations of isolated pyrenoids which had been extracted with 1% SDS before fixation showed the absence of any core or membranous material (Fig. 6). The pyrenoid starch appeared to be the same as the pyrenoid starch of unextracted, isolated pyrenoids.

A light microscope field of isolated pyrenoids stained with mercuric bromphenol blue is shown in Fig. 7. Preparations extracted with SDS were unable to bind the stain, confirming the extracting effect of SDS on the core material (Fig. 8).



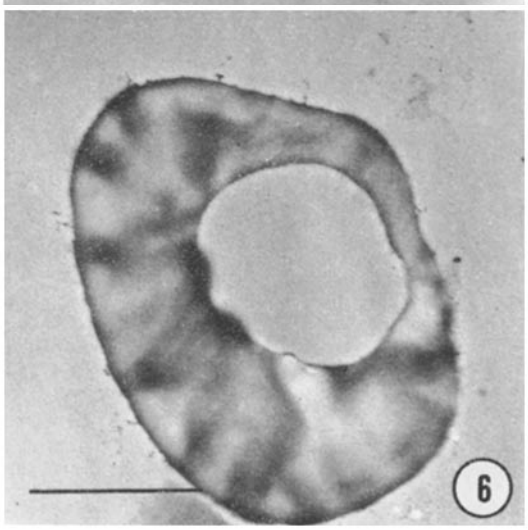


FIGURE 4 Electron micrograph of a section through an isolated pyrenoid of *Eremosphaera viridis* fixed in glutaraldehyde followed by osmium tetroxide. Note intact core. Bar at lower left represents 1 μ. × 20,000.

FIGURE 5 Electron micrograph of a section through an isolated pyrenoid of *Eremosphaera viridis* fixed in glutaraldehyde followed by osmium tetroxide. Note intact membrane in core. Bar at lower left represents 1 μ. × 38,000.

FIGURE 6 Electron micrograph of a section through an isolated pyrenoid extracted with sodium dodecyl sulfate. Note absence of all core material. Bar at lower left represents 1 μ. × 23,000.

Spectroscopy

The ultraviolet spectrum of pyrenoid extracts in SDS showed an absorption peak at 277 nm, typical of proteins (Fig. 9). The ratio of absorbance at 277 nm to the absorbance at 257 nm was found to average 1.71 ± 0.33 for nine different samples of extract.

In the CsCl-sucrose gradient the pyrenoid zone appeared light green while the stroma-starch band immediately below it appeared white. Upon extraction of the pyrenoid fraction with SDS followed by centrifugation to remove the starch, the light green color was left in solution while the pellet of pyrenoid starch was white. The visible-light absorption spectrum of the solution showed peaks or shoulders at 671, 650, 610, 540, 470, 440, 417, and 330 nm. The absorption spectra of 100–200-fold dilutions of solubilized chloroplast membranes also showed peaks and shoulders at these wavelengths.

Starch and Protein Analysis

The ratio of the total number of grains of stroma-starch to the total number of pyrenoids recovered from the gradients varied from 1.7 to 4.1, depending on the experiment. These values probably reflect differences in the "physiological ages" of the cultures used in the experiments, the older cultures typically having more stroma-starch.

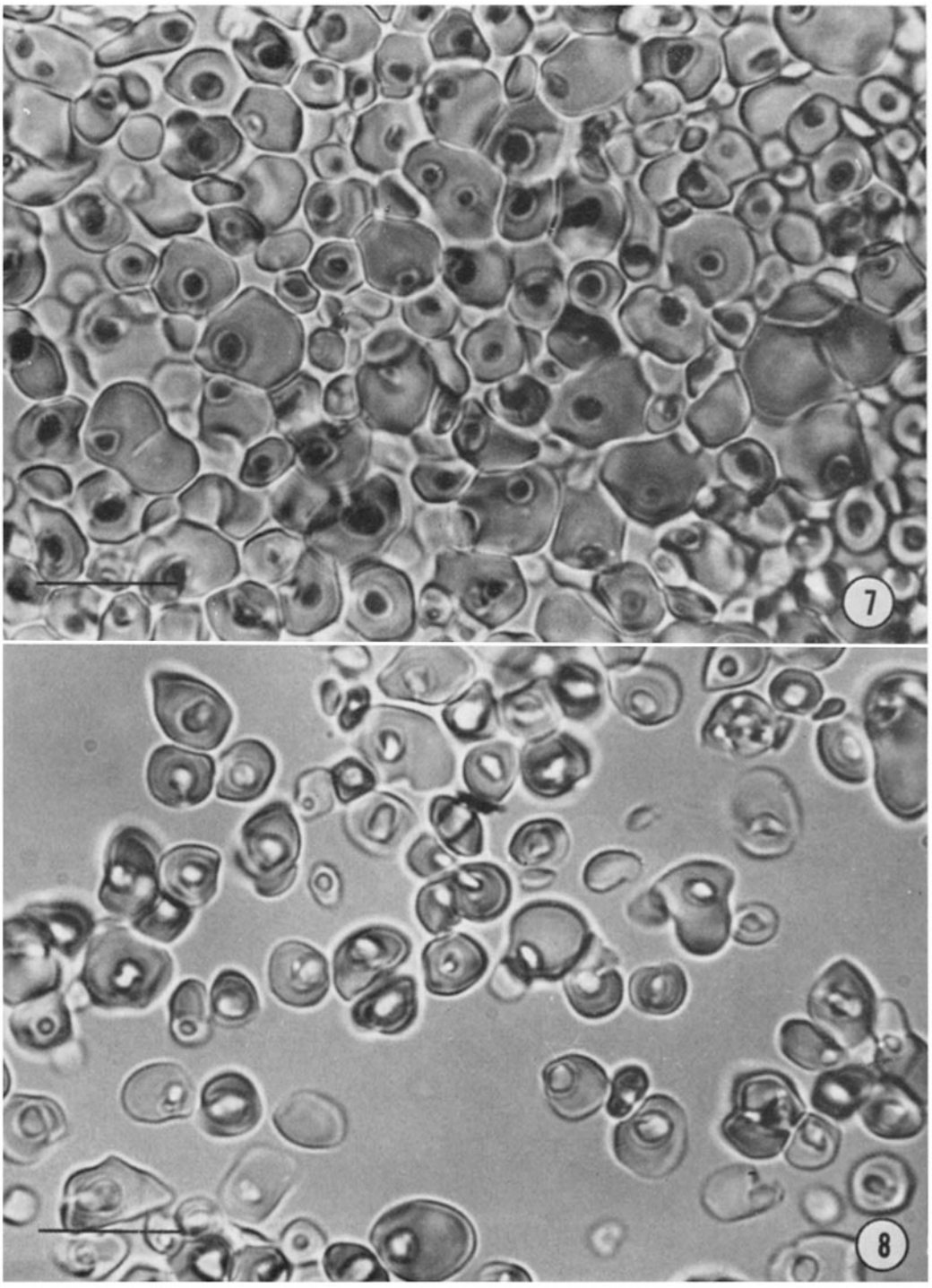


FIGURE 7 Light micrograph of a field of isolated pyrenoids fixed in glutaraldehyde and stained with mercuric bromphenol blue. Bar at lower left represents 10 μ. $\times$ 2220.

FIGURE 8 Light micrograph of a field of isolated pyrenoids following extraction with sodium dodecyl sulfate, fixation with glutaraldehyde, and staining with bromphenol blue. Note absence of core material. Bar represents 10 μ. $\times$ 2220.

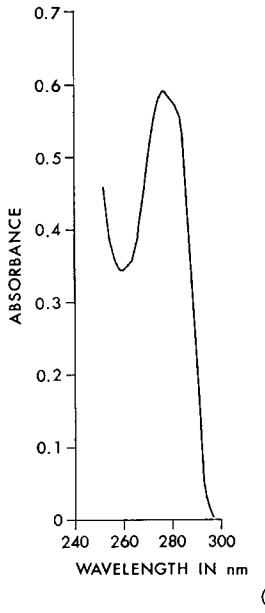


FIGURE 9 Ultraviolet absorption spectrum of pyrenoid extract. Extract contained sodium dodecyl sulfate to solubilize the pyrenoid core.

The pyrenoid fraction was composed of 92–98% pyrenoids by particle count, the remainder being stroma-starch. The stroma-starch fraction was 85–90% stroma-starch, the remainder being pyrenoids. Since there was some stroma-starch in the pyrenoid fraction and vice versa, the amount of protein per pyrenoid and per grain of stroma-starch could best be estimated by a method which incorporated all of the data from both fractions. This is accomplished by solving the following equations for x and y:

$$P_p x + S_p y = T_p$$

$$P_s x + S_s y = T_s$$

where P_p and P_s are the total numbers of pyrenoids in the pyrenoid fraction and stroma-starch fraction respectively; S_p and S_s are the total numbers of grains of stroma-starch in the pyrenoid and stroma-starch fractions respectively; and T_p and T_s are the total amounts of protein in the pyrenoid and stroma-starch fraction respectively. All of these values were readily determined. The unknown x (the amount of protein per pyrenoid) was found to average $1.63 \pm 0.46 \times 10^{-9}$ mg, and the unknown y (the amount of protein per grain of stroma-starch) was found to average $0.02 \pm 0.10 \times 10^{-9}$ mg for three replicate experiments.

By using a set of equations similar to those above, it is possible to determine the starch content per pyrenoid ($39.2 \pm 9.6 \times 10^{-9}$ mg) and per grain of stroma-starch ($27.3 \pm 9.2 \times 10^{-9}$ mg). Due to the wide variation in size of both the pyrenoid and stroma-starch grains, it is difficult to interpret these values.

Electrophoresis

Photographs of electrophoresis gels run with either pyrenoid or chloroplast extracts are shown with their respective densitometer tracings in Fig. 10. In all, at least 16 different bands could be seen in the pyrenoid fractions. Many of these bands, however, were very faint and not evident in all of the gels of each preparation. Of the sixteen, two major bands, labelled *h* and *p* in Fig. 10, together contained about 89% of the total protein-stain complex of each gel. Specifically, protein *h* contained 58% and protein *p* contained 31% of the total protein. The ratio by weight of protein *h* to protein *p* is approximately 2:1, assuming that the amount of stain in a given band is directly proportional to the amount by weight of protein in that band. The molecular weight estimates of these proteins as shown in Fig. 2 are 59,000 for *h* and 12,300 for *p*. In addition to *h* and *p* three other bands, designated *i*, *j*, and *k* in Fig. 10, could always be accounted for in the gels. These proteins had estimated molecular weights of 54,000, 50,000, and 45,000, respectively, and composed 3, 3, and 2% of the total protein on the gels.

The two major peaks of the chloroplast fragment extract, designated c_1 and c_2 in Fig. 10, have estimated molecular weights of 21,000 and 19,000, respectively. A minor band frequently found in gels of the pyrenoid extracts corresponded in position to these major bands of the chloroplast gels. Some of the minor bands seen on the chloroplast gels corresponded to bands *h*, *i*, and *j* and sometimes *p* of the pyrenoid extracts. It is assumed that these bands are due to pyrenoids contaminating the chloroplast preparation.

One band of pigment was evident in gels of both pyrenoid and dilute chloroplast extracts. This band of pigment, which had an Rm value of 1.21, did not bind the Coomassie stain, however, and was largely lost during the destaining process, indicating that there was no protein complexed with the pigment. On gels which had a relatively

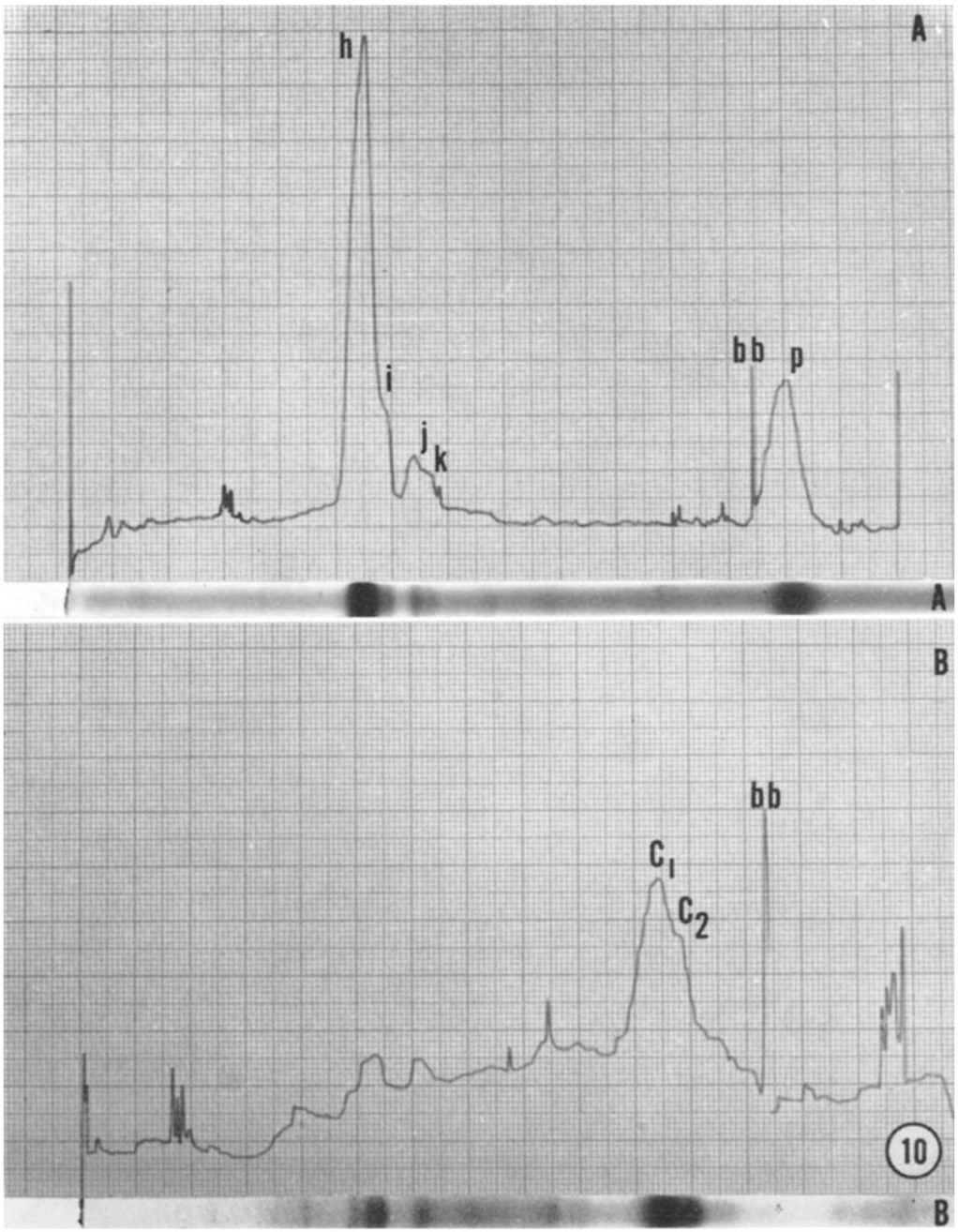


FIGURE 10 Photographs of electrophoresis gels and their respective densitometer tracings of a pyrenoid (*A*) and a chloroplast fragment (*B*) preparation. Direction of migration of the protein was from left to right. *bb*, point of migration of the bromphenol blue marker; *h*, *i*, *j*, *k*, *p*, major pyrenoid proteins; c_1 and c_2, major chloroplast fragment proteins.

high concentration of membrane extract, the pigment moved with the protein. In these cases, however, pigment and protein formed a broad zone in the lower half of the gels rather than forming discernible bands.

Enzyme Assays

Before grinding for the enzyme assay, the pellet of pyrenoids and stroma-starch had an homogeneous light green color. After grinding and centrifuging, the pellet appeared white with a thin layer of green material on top. The green layer was composed of granular and membranous material as judged by light and electron microscopy. A sample of the ground pellet fixed in glutaraldehyde and stained with mercuric bromphenol blue showed that much of the core material had been released from the pyrenoids during grinding.

The assay for RudP carboxylase showed that the specific activity of pyrenoid extracted material was from 14 to 57 times greater than the specific activities of either the chloroplast fragment preparation or the homogenization supernatant which contains the total soluble protein of the cell (Table I). Approximately 40% of the total carboxylase activity recovered is contained in the pyrenoids, most of the remaining being found in the homogenization supernatant. Mixtures of the various enzyme preparations have approximately additive activities. Control assays, which lacked substrate, enzyme preparation, or assay enzymes, all showed no reaction. The sample of pyrenoid extract which had been heated showed a complete loss of carboxylase activity.

In addition to RudP carboxylase, the pyrenoid extract also contained both ribose-5-phosphate isomerase and ribulose-5-phosphate kinase activities as judged by the ability of pyrenoid extract to fix CO_2 using ribose-5-phosphate as a substrate instead of ribulose-1,5-diphosphate. The level of activity of these enzymes was low, however.

Electrophoresis gels of the enzyme samples showed that all three preparations contained protein *h*. These gels contained little protein, however, and other pyrenoid proteins were not visible in the gels. The chloroplast sample showed a band at the position of the c_1 and c_2 proteins while the homogenization supernatant contained many bands which were not characterized further.

TABLE I

*Specific Activities of RudP Carboxylase from Various Fractions of Eremosphaera viridis**

	Source of enzyme		
	Pyrenoid	Chloroplast fragments	Homogenization supernatant
Experiment 1	0.157	0.011	0.004
Experiment 2	0.114	0.005	0.003
Experiment 3	0.344	0.007	0.006

* Units given are defined in terms of μM CO_2 fixed/min per mg protein. The assay used was that described by Racker.

DISCUSSION

The success in isolating pyrenoids was due largely to the nature of the experimental material. *Eremosphaera viridis*[2] proved to be an excellent choice, for several reasons. The cells are large and easily ruptured by relatively gentle means releasing, thereby, hundreds of chloroplasts and pyrenoids per cell. The large cell walls are easily removed from the homogenate by filtration. The pyrenoids have a solid shell of dense starch around the protein core which protects the core material during homogenization and provides a "weight" about the protein, making separation relatively easy.

All of the peaks of the absorption spectrum of pyrenoid extracts, except the one at 330 nm, can be accounted for as being characteristic of a mixture of chlorophyll-a and chlorophyll-b (66). The nature of the peak at 330 nm is not known. The fact that the chlorophyll freed from the pyrenoid by physical grinding is associated with membranous material supports the view of Gibbs (22) and Brown et al. (10) that the chlorophyll is contained in the membranes which traverse the pyrenoid core. The SDS extracts of the chloroplast fragments showed an absorption spectrum similar to that of the pyrenoid extracts, indicating that with respect to pigment content the membranes in the pyrenoid are qualitatively similar to the thylakoids in the rest of the chloroplast.

The molecular weight estimates of the two major proteins of the chloroplast fragments are similar to some others reported in the literature

[2] The author is greatly indebted to Dr. Harold Bold of the University of Texas for suggesting the use of *Eremosphaera* as the material of choice for pyrenoid solation.

for the "structural protein" of chloroplast membranes solubilized by detergent (14, 15, 30). It is evident by the difference in their electrophoretic banding patterns, that the major pyrenoid proteins do not contribute directly to these major structural proteins of the chloroplast membranes.

The Abs_{277}/Abs_{257} ratio of pyrenoid extracts suggests the absence of nucleic acid; however, small amounts of nucleic acid would probably not be detected by this method.

That protein is a major constituent of the pyrenoid core material of *Eremosphaera viridis* is supported on several grounds. Isolated pyrenoids stain strongly with mercuric bromphenol blue which is considered to be a protein specific stain. The extracts of pyrenoid core material show a single absorption maximum at a wavelength of 277 nm, typical of proteins. The material moves and stains as a protein in electrophoresis and gives a positive reaction with Folin's reagent during protein determinations. An average value of 1.63×10^{-9} mg protein/pyrenoid does not seem unreasonable in light of a total soluble protein per chloroplast value for higher plants of about 4×10^{-9} mg (46). Taking 1.19 g/ml as a reasonable density for protein (40), a sphere of protein which has a weight equal to 1.6×10^{-9} mg, that of the average pyrenoid, would have a diameter of 1.4 micrometer. This is close to the 2.1 micrometer–measured diameter of the average pyrenoid core.

That a major component of the pyrenoid core is fraction I protein is supported by both its physical characteristics and its enzymatic activities. In the literature, fraction I protein is usually identified by its ultracentrifugal properties and the molecular weight of the undissociated protein (34). Although these data were not obtained here, other characteristics of fraction I protein reported in the literature allow a comparison with pyrenoid protein. Both proteins are found within the chloroplast. The electrophoretic banding pattern of fraction I protein in SDS-containing gels shows two major, widely separated protein bands as does pyrenoid protein (52, 58). Moon and Thompson (47) have dissociated fraction I protein with 8 M urea and have estimated the molecular weights of the two subunits to be 54,000 and 16,000 by their elution properties on Sephadex 200 columns. These molecular weights are similar to those estimated for the two major proteins of pyrenoids (59,000 and 12,300). The ratio by weight of the larger subunit to the smaller subunit of fraction I protein has been reported to be 2:1, the same as the ratio of the larger to the smaller protein of pyrenoids (33, 52). Enzymatically, fraction I protein has been shown to have RudP carboxylase activity with which are associated, in crude preparations at least, both ribose-5-phosphate kinase and ribulose-5-phosphate isomerase activities (45, 49, 62). The same is true of pyrenoid protein. Specific activities of two purified samples of higher plant RudP carboxylase have been reported to be 1.26 and 1.42, or about five times those found for the preparations described here (49, 60).

Further support for the conclusion that pyrenoid protein contains a high concentration of fraction I protein comes from the work of Goodenough and Levine (24) and Togasaki and Levine (59) who have described a mutant strain of *Chlamydomonas* which has a greatly reduced rate of synthesis of RudP carboxylase. In this strain the pyrenoid is quite rudimentary, most of the core material being absent.

The fact that most pyrenoids have intact cores when isolated suggests that the carboxylase activity found in the homogenization supernatant cannot all be accounted for by pyrenoid protein released during the preparative process. It seems likely, therefore, that the pyrenoid is not the only place in the cell where RudP carboxylase is located. Howell and Moudrianakis (31) have reported that RudP carboxylase is found associated with the chloroplast membranes in spinach, and it is possible that this is also the case with algae. It is also possible that RudP carboxylase is found freely soluble in the chloroplast stroma (48). It is interesting to note that condensed states of fraction I protein, called stromacentres, have been found in higher plants (27), which suggests that fraction I protein can exist in both condensed and dispersed states.

The presence of RudP carboxylase and possibly other enzymes of the Calvin-Bassham cycle in the pyrenoid may explain some of the structural relationships observed with the light and electron microscopes. The close association of starch with the pyrenoid protein of green algae has led many investigators to the conclusion that pyrenoids contain the enzymes responsible for starch synthesis (9, 21, 55). The presence of a starch synthetase complex in the pyrenoid is possible but is not essential to explain the pyrenoid association of starch in green algae. An alternative explanation

is that the region just outside the pyrenoid contains a high concentration of soluble carbohydrate due to the activities of the carbon-fixing enzymes of the pyrenoid. The starch synthetase, which could be located in the stroma, acts through some intermediate to condense this carbohydrate onto the outer surface of the starch grain. A stromal localization of the starch synthetase would allow starch to be formed in other parts of the chloroplast (stroma-starch) using products of stroma or membrane-located carbon-fixing enzymes and would explain why starch grains are only very rarely seen embedded within the pyrenoid protein itself. That both carbon-fixing and starch-synthesizing enzymes are located in other parts of the chloroplast explains why algae which lack pyrenoids are still able to form starch reserves (36, 63, 65).

In Euglenophytes, Phaeophytes, and Chrysophytes the carbohydrate reserves are located in the cytoplasm of the cells outside the chloroplast. These reserves may be physically associated with pyrenoids but are spearated from them by one or all of a series of membranes including the chloroplast envelope, a layer of chloroplast endoplasmic reticulum, and a vesicle membrane which surrounds the reserve products (5, 18, 38, 41, 61). If the pyrenoid protein were directly synthesizing the polysaccharide reserves in these cases, it would have to be operating through the membranes. If, however, the area of the pyrenoid were rich in soluble carbohydrate which could easily traverse these membranes, one might expect this region to be one in which reserve products could be efficiently synthesized by cytoplasic or vesicle-bound synthetases. The protuding or stalked nature of the pyrenoids of many of these algae as well as "fingers" of cytoplasm which extend deep into the protein core have suggested to some that the pyrenoid serves as an intermediary of metabolite distribution from the chloroplast to the cytoplasm (5, 22, 41, 42). This view is consistent with the hypothesis presented here. This same general principle might also apply to the Rhodophytes, which have cytoplasmically located "starch" reserves which are not usually pyrenoid associated (8).

Although composed in part of protein generally associated with the membranes and stroma, the pyrenoid has an organization and structure of its own, and many questions about its organization, chemistry, and function remain unanswered. Possibly, the pyrenoid serves as a storehouse of the Calvin cycle enzymes to provide recently divided cells a ready source of those enzymes, which are so critical to cell growth. It will be necessary, however, to explore these questions further before the nature of algal pyrenoids can be fully understood.

The author is deeply indebted to Professor William P. Jacobs for his suggestions and comments during the preparation of this work and to Dr. Frederick Meins for his helpful suggestions for the revision of the manuscript. The author would also like to express his deep appreciation to Dr. Masayori Inouye who gave freely of his time and knowledge during the course of the research.

This research was supported by funds from grant 5-T01-GM457 of the National Institutes of Health, grant NONR 1845 (45) to Dr. W. P. Jacobs from the Office of Naval Research, and grant GB-6029 to Dr. W. P. Jacobs from the National Science Foundation. Gratefully acknowledged also is the use of the central equipment facilities of the Biology Department, Princeton University, supported by the Whitehall Foundation and the John A. Hartford Foundation.

Received for publication 16 March 1971, and in revised form 23 June 1971.

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