SOLUBILIZATION AND PROPERTIES OF CHLOROPLAST LAMELLAR PROTEIN

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An understanding of the detailed mechanisms of the chloroplast-mediated reactions of photosynthesis requires information on the components of the chloroplast as thorough as the information established for the mitochondrion.1

Two procedures for solubilization of chloroplast lamellar protein have been reported. Weber2 extracted chloroplasts with methanol and ether and dissolved the remaining protein in anhydrous formic acid to obtain subunits of approximately 165,000 particle weight. Criddle and Park3 found that a combination of 0.1 per cent sodium dodecyl sulfate and 1 per cent urea yielded solubilized protein from an acetone powder of chloroplast fragments.

In this communication, we report a method for solubilizing two thirds of the total chloroplast lamellar protein by butanol extraction and discuss some of the properties of this isolated protein. The protein can be obtained in aqueous solution without the presence of detergent or other surfactant material.

Materials and Methods.—Swiss chard (Beta vulgaris), variety dark green, was grown in the greenhouse or in a plant growth chamber and nourished with Hoagland and Arnon's4 mineral medium. Light-grown field corn (Zea mays) was grown similarly; etiolated corn plants were grown in complete darkness, and the leaves were harvested 10–12 days after planting. C14-labeled chard material was obtained from plants grown in a closed system in the presence of C14O25.

Chloroplast fragments were prepared by a method similar to that of Park and Pon.6 Two hundred gm of leaves, separated from the petioles and mid-ribs, were homogenized in a Waring Blender at 4°C with 200 ml of 0.05 M Tris buffer (titrated to pH 8 with ascorbic acid) containing 0.01 M EDTA and usually 0.5 M sucrose. Debris was removed by filtration through six layers of cheesecloth, and the green juice was centrifuged for 3 min at 1000 g. The precipitate was discarded, and the supernatant was centrifuged for 12 min at 4000 g to bring down the chloroplasts. The chloroplasts were washed extensively with 10–2 M pH 7 phosphate buffer (this ruptured the chloroplasts) and then with distilled water to remove all soluble proteins. After the material from 200 gm of leaves was re suspended in 50 ml of distilled water, it was further disintegrated in a Raytheon 10-ke sonic oscillator for 15–20 min, and the volume was adjusted to 100 ml with washings. Starch granules were separated from the lamellar fragments by centrifuging for 30 min at 9000 g.

Preparation of micelles: One gm of asolectin, a commercial phospholipid preparation from soybeans (Associated Concentrates, Woodside, N.Y.), was sonicated for 30 min in 50 ml of a solution of 0.01 M Tris buffer, pH 8, 0.25 M sucrose, and 5 × 10–4 M EDTA2 to prepare micelles. The micelles could be prepared as well by disrupting in distilled water or dilute cacodylate buffer.

Chard leaf lipid was obtained by extracting the leaves with alcohol-ether or chloroform-methanol,4 and leaf lipid micelles were obtained by sonication of the dried, extracted material in sucrose-Tris buffer-EDTA, distilled water, or dilute cacodylate buffer. Some chlorophyll, isolated from chard leaves by the procedure of Jacobs et al.8 or that of Anderson and Calvin9 somewhat modified, could also be obtained in micellar suspension by sonication for 45 min.

Assay procedures: Protein was assayed according to the Folin method of Lowry et al.11 Total nitrogen was determined by semimicro Kjeldahl digestion, followed by quantitative steam distillation and determination of ammonia with Nessler's reagent.12 The method of Chen et al.13 was used to determine phosphorus.

Chlorophyll was assayed according to the spectrophotometric method of Arnon.14 The amount
of chard lipid was estimated roughly by determining the chlorophyll content and using the ratio of chlorophyll to total leaf lipid found by Zill and Harmon\textsuperscript{18} for spinach.

C\textsuperscript{14} labeled protein was added to Butler's\textsuperscript{16} solution, and radioactivity was measured on a liquid scintillation counter. The soluble protein generally did not precipitate or form a film when added to the scintillation mixture. Radioactivity also was determined by plating with 1% gelatin in 0.01 M NaOH\textsuperscript{17} and counting with a thin-window gas-flow counter. These two methods of determining radioactivity checked well. P\textsuperscript{32} also was determined by counting with the thin-window counter.

Amino acid analysis: Protein was hydrolyzed in an evacuated and sealed glass tube with 6 N HCl for 22 hr at 115°C and 6 lb pressure in an autoclave. The amino acids were analyzed with a Beckman-Spinco amino acid analyzer.\textsuperscript{18, 19} Cysteine and cystine were determined as cysteic acid,\textsuperscript{20} and tryptophan was estimated by the method of Dreze.\textsuperscript{21} One complete amino acid determination was made for each protein isolated.

Results.—Preparation of soluble chloroplast lamellar protein by butanol extraction: A substantial portion of chloroplast lamellar protein could be solubilized by one of the methods used successfully for the isolation of lipoprotein complexed enzymes.\textsuperscript{22} Butanol extraction was performed in the following manner (Fig. 1): 55 ml of sonicated chloroplast suspension was placed in a 500-ml Erlenmeyer flask, and to this was added 20 ml of 0.2 M glycine buffer, pH 9, usually a few mg of sodium dithionite, and water to a total volume of 200 ml. (The dithionite was added to reduce cytochromes,\textsuperscript{3, 23} but it did not appear to make any difference in the protein isolation.) While the solution was stirred vigorously with a magnetic stirring bar, 50 ml of n-butanol was added slowly through a separatory funnel whose stem was drawn out to a small bore and was slightly curved so that it touched the side of the flask. The butanol was added over a period of 20–30 min at room temperature, and the mixture was stirred for an additional 45–60 min.

The emulsion was centrifuged at 15,000 g for 30 min. The upper phase (butanol) was dark green and the lower phase (aqueous) was light green or yellow green; the insoluble residue was at the interface. The butanol layer and the interfacial residue were removed by suction, and the aqueous phase, which contained the solubilized lamellar protein, was dialyzed for a few hours against 0.1 per cent sodium dodecyl sulfate (SDS) or 10\textsuperscript{−5} M buffer (see below) to remove most of the dissolved butanol.

The protein solution was removed from the dialysis bags, glycine was added, and butanol extraction was carried out as before. After the second extraction, the aqueous portion was considerably lighter yellow with a tinge of green. Usually, the entire

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Summary of method for preparation of soluble chloroplast lamellar protein.}
\end{figure}
extraction process was repeated a third time. Thus, the total treatment included three butanol extractions followed by centrifugation, removal of the nonaqueous portions, and dialysis of the aqueous material. The resultant solution of about 225 ml vol was light yellow, and usually contained about 400–500 μg protein per ml. Two thirds of the total lamellar protein can be solubilized in this manner, and 160–200 mg protein per 200 gm chard leaf was usually obtained.

The aqueous portion from the final extraction was dialyzed extensively to remove all butanol and glycine. In early experiments, the protein was dialyzed against a solution of 0.1 per cent SDS in water. Later, it was found that detergent was not required to keep the protein in solution, but that dialysis against a dilute solution of pH 6.8 cacodylate buffer, pH 7.0 phosphate buffer, or pH 8.0 Tris buffer worked equally well. The concentration of buffer used was 10⁻³ M. Dialysis could be performed at room temperature or at 4° without apparent change in the properties of the protein. When the protein solution was dialyzed against distilled water, however, 89–96 per cent of the protein came down as a yellow precipitate. About 90 per cent of the protein precipitated from the dilute cacodylate solution when sodium cacodylate was added to a concentration of 0.15 M.

Most of the experiments were performed with solubilized protein dialyzed against 10⁻³ M cacodylate buffer. Fifteen per cent sucrose was added to prevent precipitation of the protein during storage at 4° or at −20°.²⁴

Identification of protein: The yellow aqueous solution gave a positive Folin test and a positive biuret reaction. Total nitrogen analysis of a Kjeldahl digest of cold 5 per cent trichloroacetic acid-treated material indicated a concentration for protein very close to that estimated with the Folin reagent.

Protein pigment: A number of attempts were made to prevent the introduction of yellow color into the protein preparation and to remove material responsible for the color; however, none of these attempts were successful. A colorless solution could not be obtained by adding ascorbate, menadione, dithionite, or dithiothreitol during extraction, nor by performing the butanol extraction under a nitrogen atmosphere. Organic solvents did not remove the chromophoric material from the solubilized protein, nor was the color separated from the protein by partial precipitation with ammonium sulfate.

Spectra of the solubilized protein are shown in Figures 2 and 3. For Figure 2, the material was extracted only once with butanol, and it gave peaks in the visible region at 406, 541, 611, and 660 μm. This spectrum is similar to that obtained by Weber² with lamellar protein from Antirrhinum majus dissolved in anhydrous formic acid. The peaks are characteristic of pheophytins and pheophorbides.

Figure 3 shows the spectrum of protein prepared by three butanol extractions, followed by concentration to 6.7 mg protein per ml with dry Sephadex G-25. The only peak in the visible was near 404 μm; this maximum was a consistent feature of all solubilized chard lamellar protein preparations.

The amount of chlorophyll derivatives represented by these absorption maxima was estimated from the molar extinction coefficients given for the 660-μm (ε = 4.5) and 404-μm (ε = 4.9) peaks of pheophytins and pheophorbides (Organic Electronic Spectral Data, 1960). If it is assumed that all of the chlorophyll was originally bound to the solubilized protein (about two thirds of the total lamellar protein), approximately 4–5 per cent of the chlorophyll derivative remained
attached to the protein after one butanol extraction and only 0.1–0.2 per cent after three butanol extractions. Weber\(^2\) used similar calculations to estimate that 98–99 per cent of the original chlorophyll molecules were removed from his lamellar protein preparations.

**Binding experiments:** A preliminary series of experiments was performed to estimate the capacity of the solubilized protein to bind with chloroplast substances and other materials. It was observed that the addition of 131 \(\mu g\) or more of phospholipid micelles to 416 \(\mu g\) of solubilized protein (in \(10^{-3}\) \(M\) neutralized cacodylate buffer containing 15\% sucrose) prevented precipitation of the protein upon dialysis against distilled water. Prior to dialysis, the protein and micelles were mixed gently for 20 min at room temperature.

With smaller amounts of the phospholipid micelles (65 \(\mu g\) and less phospholipid per 416 \(\mu g\) protein), some precipitation occurred. Phosphate analysis and \(^{14}\)C determination of the labeled protein indicated that some phospholipid coprecipitated with a portion of the protein. The ratio of bound phospholipid to protein was very high (about 170 \(\mu g\) phospholipid phosphorus per mg protein). This extensive binding may have reflected the particular method employed for examining the protein-phospholipid interaction. Similar experiments with chard leaf lipid micelles and chlorophyll micelles also indicated a quantitatively large interaction with the protein.

When 1 mg of horse heart cytochrome \(c\) in \(10^{-3}\) \(M\) pH 6.8 cacodylate buffer was added to 416 \(\mu g\) of solubilized protein, cytochrome immediately coprecipitated with 75–85 per cent of the lamellar protein from a total volume of 4 ml. The precipitate was washed twice with dilute buffer, and it was calculated from the extinction coefficients for cytochrome \(c\) that about 0.3 mg of the cytochrome was bound per mg protein. When the two proteins were mixed and cacodylate was added after mixing to a final concentration of 0.5 \(M\), no combination was observed.

Use of \(^{32}\)P-labeled phosphate revealed that a maximum of 1–2 \(\mu g\) phosphorus was bound per mg of the solubilized protein.

**Lamellar protein from light-grown and etiolated corn:** The butanol extraction method as applied to solubilize lamellar protein from chard chloroplasts was used
successfully to obtain soluble protein from corn chloroplasts and etiolated corn proplastids.

Two butanol extractions of 32 gm of light-grown corn leaves yielded 23.2 mg of yellow solubilized protein (72.5 mg protein per 100 gm leaves); this yield was comparable to that obtained from chard. Seventy-two gm of etiolated leaves yielded 8.14 mg solubilized protein, corresponding to only 11.3 mg protein per 100 gm leaves.

The spectra of the solubilized corn proteins (three butanol extractions) are shown in Figure 4. The two spectra are quite similar and also resemble the spectrum of solubilized chard protein, although the 404-μm peak characteristic of the chard material is replaced by a 398-μm peak.

Both of the corn proteins were precipitated by dialysis against distilled water or by the addition of cacodylate buffer to 0.5 M. Experiments with chard lipid micelles and chlorophyll micelles (see Materials and Methods) showed that both the corn proteins bound these substances.

**Amino acid composition:** The complete amino acid compositions of proteins isolated from chard chloroplasts, corn chloroplasts, and etiolated corn proplastids are shown in Table 1. The similarities in composition of all three proteins are remarkable. Even more remarkable are the similarities with the values obtained by Weber for proteins obtained from four different plant sources and prepared in an entirely different manner.

In all proteins, the amounts of cysteine and histidine are particularly low. Structural protein obtained from mitochondria contains 10–40 times the amount of cysteine found for the chloroplast proteins.

**Discussion.**—The extraction of chloroplast lamellar fragments with n-butanol was found to be an effective method of removing lipid and pigment from the lamellar protein and solubilizing most of the protein. Our experiments indicate that proplastid protein can be isolated in the same manner.

There is little doubt that the protein obtained was derived from the lamellae. Soluble proteins were washed out from the chloroplast before butanol extraction; the chlorophyll extracted into the butanol layer was essentially protein-free, indicating dissociation of the in vivo protein-chlorophyll complex. Chloroplast ribosomes, located in the stroma fraction, were removed by washing.

A small percentage of the chlorophyll molecules, modified during the isolation procedure, apparently remained bound to the protein. Magnesium is easily lost from chlorophyll, and the spectrum of the chloroplast protein pigment obtained after one butanol extraction was very similar to that of pheophytin and pheophorbide. With the thrice-extracted protein, the amount of chlorophyll represented was about 0.1–0.2 per cent of that in vivo; further extraction with butanol did not remove the color. It is possible that the pigmented material which remains at-
TABLE 1
AMINO ACID COMPOSITION OF CHLOROPLAST AND PROPLASTID PROTEINS

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mole (%)</th>
<th>Four plants* (range)</th>
<th>Chard† chloroplast</th>
<th>Corn‡ chloroplast</th>
<th>Corn‡ proplastid</th>
<th>Beef heart§ mitochondria structural protein</th>
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<tr>
<td>Aspartic acid</td>
<td>7.6-8.9</td>
<td>7.9</td>
<td>7.7</td>
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<tr>
<td>Threonine</td>
<td>4.8-6.1</td>
<td>4.9</td>
<td>5.4</td>
<td>5.6</td>
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<td>Serine</td>
<td>6.0-6.8</td>
<td>5.8</td>
<td>5.7</td>
<td>6.7</td>
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<tr>
<td>Glutamic acid</td>
<td>8.1-8.4</td>
<td>8.1</td>
<td>7.9</td>
<td>8.5</td>
<td>6.2</td>
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<td>5.2</td>
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<td>Methionine</td>
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<td>4.4</td>
<td>4.4</td>
<td>5.0</td>
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<tr>
<td>Leucine</td>
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<td>13.0</td>
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<td>Tyrosine</td>
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<td>2.7</td>
<td>2.9</td>
<td>3.2</td>
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<td>Phenylalanine</td>
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<td>5.1</td>
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<tr>
<td>Amide-NH₂</td>
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<td>4.4</td>
<td>4.7</td>
<td>6.4</td>
<td>6.3</td>
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<tr>
<td>Lysine</td>
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<td>1.4</td>
<td>1.2</td>
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<td>Histidine</td>
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<td>3.8</td>
<td>4.5</td>
<td>4.3</td>
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<tr>
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<td>1.8</td>
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<tr>
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<td>0.5</td>
<td>0.5</td>
<td>0.4</td>
<td>7.7</td>
<td></td>
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* From Weber. Plants are Chlorella pyrenoidosa, Allium porrum, Spinacia oleracea, and Antirrhinum majus.
† Twice-extracted with butanol.
‡ Twice-extracted with butanol.
§ From Cridle et al.*

The interaction of various substances with the solubilized proteins was tested to gain an insight into the scope of the binding capacity of the isolated material. The stoichiometry of binding was not determined with precision, but much phospholipid, chard lipid, and chlorophyll were bound. Little inorganic phosphate was bound, and cytochrome c coprecipitated with the protein. The nature of the binding interactions is unknown.

It is interesting to note that the protein isolated from proplastids has characteristics similar to those of the chloroplast lamellar protein. From the similarity in the absorption spectra of the two corn proteins, it is likely that their chromophoric groups are similar; the group on the isolated proplastid protein could be a modified precursor of chlorophyll. The similarities in amino acid composition of the two proteins suggest that this proplastid protein plays a functional role in the developed chloroplast.

Summary.—Most of the protein of chard and corn chloroplast lamellae and corn proplastids was solubilized by treatment with n-butanol at room temperature. No detergent was required to solubilize the proteins. The solubilized proteins interacted with large amounts of chloroplast lipid and chlorophyll, and tests with the chard protein showed that the protein could also interact with phospholipid and cytochrome. The amino acid compositions of all the proteins were remarkably similar.

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