Sulfolipid Localization in Lamellar Lipoprotein

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The plant sulfolipid, 6-sulfo-a-β-quinoovopyrano-syl-(1 → 1')-2',3'-di-O-acyl-α-glycerol, has been found in high concentrations in all and only in photosynthetic O₂-producing organisms yet investigated (5, 6). Shibuya and Hase (21) recently demonstrated the concerted changes in chloroplast development and sulfolipid content in a green alga, Chlorella protothecoides. It is increasingly apparent that these surfactant lipids play an important role in chloroplast lipoprotein structure. For an intrinsic study of biochemistry of the sulfolipids, the localization of the compounds in the organelle is of obvious interest. The intracellular distribution of the sulfolipid has been studied (29, 30) but the difficulty of quantitatively isolating chloroplasts from photosynthetic tissues precluded assignment of their distribution within the cell on the basis of radioactivity measurements.

In the present study, a radiochemical investigation on the distribution of various components was first conducted with the chloroplasts isolated from C¹⁴-labeled Lemma perpusilla. Since the lamellar fraction was found to represent practically whole chloroplasts, as far as the glycolipid contents were concerned, it seemed desirable to develop a procedure for the quantitative isolation of such chloroplast fragments from whole plant tissues. A radiochromatographic investigation of an S⁸⁵-labeled preparation thus obtained has demonstrated the specific localization of the sulfolipids in the lamellar fragments. A particulate fraction from chloroplasts first described by Park and Pon (19, 20) as containing pigments and part of the electron transport systems for photosynthesis.

This paper reports the details of these experiments as well as of the chemical characterization of a spinach lamellar preparation, and discusses significance of the present findings.

Material and Methods

Lemma perpusilla Uniformly Labeled with C¹⁴ or S⁸⁵. Lemma perpusilla 6746, original strain supplied by Professor S. Tamura, University of Tokyo, was grown under photosynthetic conditions in a synthetic medium (12). Uniform labeling of this water plant with radiocarbon was achieved by growing it in C¹⁴O₂ for 1 week during which the number of plants increased approximately 10-fold (16). Lemma uniformly labeled with S⁸⁵ was prepared by culture in a low-sulfur medium, in which most of the sulfate in the synthetic medium (12) was replaced by nitrate, containing S⁸⁵O₄⁻ (700 μC/potom S) under photosynthetic conditions for 4 days. The plants increased in number 3-fold during the culture.

Preparation of Chloroplasts and Their Fragments from Lemma-C¹⁴. Chloroplasts and chloroplast fragments were prepared from Lemma perpusilla uniformly labeled with C¹⁴ using a modification of the Park and Pon (19) procedure. Lemma-C¹⁴ was harvested by filtration and washed with a sucrose-phosphate buffer, pH 7.4, which contained 0.5 M sucrose, 0.1 M potassium phosphate and 10⁻² M EDTA. Approximately 15 Lemma plants were ground in a chilled mortar with 2 ml of the sucrose-phosphate buffer and transferred to a centrifuge tube with 3 ml of the same buffer. The homogenate was centrifuged at 200 g for 5 minutes and the precipitate was again ground with 5 ml of the sucrose-phosphate buffer, and recentrifuged. The green precipitate was designated as 0.2 P. The combined supernatant liquid was centrifuged at 600 g for 12 minutes. The precipitate was once washed by resuspending it in 2 ml of 0.5 M sucrose and centrifuging at 600 g for 12 minutes. The precipitated chloroplasts were suspended in 4 ml of 10⁻³ M potassium phosphate buffer, pH 7.4, and sonically ruptured at 9 kc for 90 seconds. The sonicate was transferred to a centrifuge tube with additional 3 ml of the 10⁻³ M buffer and centrifuged at 14,000 g for 10 minutes. The green supernatant was centrifuged at 110,000 g for 10 minutes. The precipitate was designated as 110 P. The supernatant liquid was again centrifuged at 145,000 g for 30 minutes. The supernatant and the precipitate were called 145 S and 145 P, respectively. 145 P was dark green and 145 S was completely colorless. All operations were carried out at or near 0°C.

Quantitative Isolation of Lamellar Membrane Fraction from Lemma-S⁸⁵. Lemma perpusilla-S⁸⁵, repeatedly washed with water and 10⁻⁸ M potassium phosphate buffer, pH 7.4, was well ground in a mortar with a small amount of the same buffer, and then disintegrated by sonic oscillation at 9 kc for 3 minutes. The sonicate was centrifuged at 14,000 g for

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1 Received April 26, 1965.
2 This work was supported in part by grants from the Institute for Arthritis and Metabolic Diseases, United States Public Health Service, and from the National Science Foundation.
10 minutes, and the precipitate was resuspended in the same buffer and centrifuged at 14,000 g for 10 minutes. This resuspension of the precipitate and centrifugation were further repeated 4 times until the precipitate (14 P) became completely colorless. The combined supernatant liquid was centrifuged at 145,000 g for 30 minutes. The dark green precipitate was washed once with water and collected by re- 
centrifugation (145 P). The supernatant liquid (145 S) was completely colorless. All operations were carried out at or near 0°C.

Radiochromatographic Analyses of Lemma Components. Cellular components of Lemma-C14 were separated into macromolecules, water-soluble low-
molecular weight compounds and individual lipids by an ion-exchange resin-paper chromatography on Amberlite WB-2 (Rohm and Haas Company, Philadelphia) with the Marinetti solvent (13), disobutyl ketone-acetic acid-water (8:5:1, v/v/v). S85- 
Labeled components were chromatographed first with phenol-water (500:130, w/w) and second with butanol-propionic acid-water (142.7:100, v/v/v) (1). The solution or the suspension to be analyzed was directly applied on the origin of the chromatograms without previous extraction in order to avoid possible fractionation and oxidation of lipids. Quantitative extraction of all soluble components was effected with the first developing solvents as chromatography proceeded.

Identification of the compounds separated by chromatography was carried out by means of co-
chromatography and coelectrophoresis on paper of the eluted materials or their deacylation or hydrolysis products with authentic compounds. Radioactivity of the spots on chromatograms was measured directly with 5-cm diameter end-window Ge-M-1 tube (Type 5006, Nikon Musen Company, Tokyo).

Preparation and Chemical Analysis of Spinach Lamellar Fragments. Fresh spinach leaves obtained from local markets were washed with cold water and blotted dry. The leaves (180 g) were homogenized in 180 ml of 0.1 M potassium phosphate buffer, pH 7.4, in a blender operated at full speed for 2 minutes. The homogenate was subjected to sonic rupture at 9 kc/sec for 3 minutes. The sonicate was filtered through 8 layers of cheesecloth. The filtrate was centrifuged at 14,000 g for 10 minutes. The supernatant liquid was then centrifuged at 145,000 g for 1 hour. The precipitate was resuspended in 30 ml of water with the aid of a Potter-Elvehjem homogenizer, and again centrifuged at 145,000 g for 30 minutes to give approximately 200 mg (dry wt) of the dark green lamellar particulate fraction.

The final precipitate was fractionated into 3 major groups: lipids, nucleic acids and proteins-poly-
saccharides, by successive extraction with ethanol, chloroform-methanol (2:1, v/v), ethanol and ace-
tone for lipids, and then 5% hot perchloric acid for nucleic acids. Lipid content was estimated by dry-
ing a portion of the lipid fraction under reduced pressure and weighing it. Nucleic acid content was de-
termined from the OD at 260 nm of the fraction assuming the OD 260 nm was 33 for 1 mg/ml solution. The protein and polysaccharide fraction, i.e. the residue of a hot perchloric acid extraction was dried at 110°C for 3 hours and weighed. Protein and polysaccharide contents in this fraction were assumed to be the amount recovered as amino acids after acid hydrolysis and the residue thereof, respectively. Chlorophylls in the lipid fraction were estimated spectrophotometrically by measuring the OD at 663, 644 and 624 nm according to the equations described by Huzisige (11). The protein and polysaccharide fraction was hydrolyzed with 6 N hydrochloric acid at 110°C for 22 hours, and its amino acid composition was determined essentially by the method of Spack-
man et al. (24) with an amino acid analyzer (Type II1; Mitamura Riken Kogyo Inc., Tokyo).

Spinach lamellar particles, quantasomes, prepared by the procedure of Park and Pon were extracted with hot 80% ethanol and hot ethanol-toluene (3:1). The combined extracts were concentrated in vacuo and taken up in chloroform. The lipids were freed of sugars and salts by passing through a Sephadex G-50 column by the method of Wells and Dittmer (28). The chloroform was replaced with ethanol and the lipids decylated during 20 minutes at 37°C with 0.1 M sodium methylate in methanol. Two-dimensional chromatograms of the products were sprayed with the periodate-SO4-Schiffs reagents to reveal glyco-
sides and glycerophosphoryl esters.

Results

The result of the Amberlite WB-2 chromatography of the 5 subcellular fractions from Lemma per-
pusilla-C14 chloroplasts is shown in table I. Since the plant was uniformly labeled with C14 by culture in
C14O2, the radioactivity of each component represented the relative molar concentration of its constituting 
carbons. The 0.2 P fraction contained large unruptured fragments and only its 80% ethanol extract could be analyzed. Since 14 P was not washed, it had a dense green color, indicating contamination by 110 P and 145 P in this fraction. The total radioactivity of 14 P, 110-145 P and 145 S was 41, 52 and 7% of the total chloroplast activity, respectively. The C14 percentages of the major glycolipids, sul-
folipid, galactosyl diglyceride, and digalactosyl digly-
eride (3, 9), in chloroplasts were 2, 3, and 5 respect-
ively. Of these activities, 86, 92 and 85% were 
found in 110 P and 145 P. Both were equivalent to the lamellar lipoprotein (8, 19); in later experiments both 110 P and 145 P were designated simply as 145 P. Although this fraction contained fragments of other membranous structures, the lipid composition reflected that of isolated chloroplasts.

The 14 P fraction from S85-labeled Lemma was cold-aged (Shibuya and Maruo, to be published) by freezing a suspension (15 mg dry wt/ml) at -30°C for 2 days. It was thawed slowly and centrifuged at 1000 x g for 10 minutes. The precipitate was re-
suspended and recentrifuged to obtain microsome-

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free lamellar fragments. The supernatant, which would contain S\textsuperscript{35}-labeled sulfolipid of microsomal or other particulate structures, contained no detectable radioactivity.

Figure 1 represents a typical autoradiogram of the lamellar particulate fraction isolated from \textit{Lemna perpusilla} uniformly labeled with S\textsuperscript{35}. Chromatographed in x-direction first, with phenol-water and y-direction second, with butanol-propionic acid-water.

![Autoradiogram of lamellar particles](image)

**Fig. 1.** Autoradiogram of lamellar particles quantitatively isolated from \textit{Lemna perpusilla} uniformly labeled with S\textsuperscript{35}. Chromatographed in x-direction first, with phenol-water and y-direction second, with butanol-propionic acid-water.

Data for the radioactivity in the subcellular fractions of \textit{Lemna perpusilla-S35} uniformly labeled with C\textsuperscript{14} are presented in Table I. The autoradiogram of 145 S. The result of the 2-dimensional radiochromatography of these 3 fractions of \textit{Lemna-S35} is shown in Table II. Since S\textsuperscript{35}-labeling of the plant was uniform the observed radioactivities represented relative molar concentrations. The sulfolipid and the lysosulfolipid were found concentrated in the lamellar particulate fraction of the plant.

fractionation and chemical analyses of the lamellar particulate fraction isolated directly from spinach leaves were carried out as described for \textit{Lemna}. The results are summarized in Table III. Amino

**Table I. Distribution of Radioactivity in Subcellular Fractions of Lemna perpusilla Uniformly Labeled with C\textsuperscript{14}

<table>
<thead>
<tr>
<th>Fraction**</th>
<th>0.2 P Ethanol extract</th>
<th>14 P</th>
<th>110 P</th>
<th>145 P</th>
<th>145 S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>Macromolecules</td>
<td></td>
<td>...</td>
<td>38,900</td>
<td>26,200</td>
<td>4100</td>
</tr>
<tr>
<td>Water-soluble low</td>
<td></td>
<td>7500</td>
<td>3020</td>
<td>3390</td>
<td>1380</td>
</tr>
<tr>
<td>M. W. compounds</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfolipid</td>
<td></td>
<td>6770</td>
<td>237</td>
<td>1660</td>
<td>364</td>
</tr>
<tr>
<td>Digalactosyl diglyceride</td>
<td></td>
<td>17,900</td>
<td>600</td>
<td>4100</td>
<td>877</td>
</tr>
<tr>
<td>Galactosyl diglyceride</td>
<td></td>
<td>10,500</td>
<td>240</td>
<td>2210</td>
<td>555</td>
</tr>
<tr>
<td>Other lipids*</td>
<td></td>
<td>58,800</td>
<td>2240</td>
<td>11,200</td>
<td>2080</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>45,200</td>
<td>48,800</td>
<td>9360</td>
<td>7820</td>
</tr>
</tbody>
</table>

* Other lipids included chlorophylls, triglycerides, lysosulfolipid and phospholipids.

** Data are for particles from isolated chloroplasts except for fraction 0.2 P.

**Table II. Distribution of the Sulfolipids in Lemna perpusilla-S\textsuperscript{35}

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>14 P</th>
<th>145 S</th>
<th>145 P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfolipid</td>
<td>S\textsuperscript{35} cpm</td>
<td>570</td>
<td>1980</td>
</tr>
<tr>
<td>Lysosulfolipid</td>
<td>S\textsuperscript{35} cpm</td>
<td>0</td>
<td>335</td>
</tr>
</tbody>
</table>

* Particulate fractions were isolated directly from the homogenate.

**Table III. Characterization of Spinach Lamellar Fragments*

<table>
<thead>
<tr>
<th>Lipid**</th>
<th>Weight %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophylls***</td>
<td>10.1</td>
</tr>
<tr>
<td>Protein†</td>
<td>46.1</td>
</tr>
<tr>
<td>Polysaccharide</td>
<td>17</td>
</tr>
<tr>
<td>Nucleic acid††</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Purified by cold-aging (freeze-reaggregation technique) to separate from microsomal particles.

** Extracted by methanol and chloroform-methanol and dried at reduced pressure under nitrogen.

*** Calculated from absorption at 578 nm according to Warburg.

† Residue after extraction of lipid and nucleic acid. N = 15.32 %, C = 48.49 %, H = 6.62 %, S = 0.

†† Calculated from absorbance at 200 nm in hot perchloric acid extract.

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acid composition of the protein moiety of the preparation is shown in Table IV.

Decacylation of spinach lamellar lipids revealed 4 main products. The most predominant were mono- and digalactosyl glycerol. Approximately equal amounts of diglycerophosphate (GPG) and sulfoquinovosyglycerol (GQS) were observed. Although the method is sensitive to glycerophosphoryl choline (GPC), none (i.e. less than 5% of total lipids) was observed. A small amount of diglycerophosphoryl glycerol (GPG) was detected. The sulfolipids were accurately determined. The direct separation of cellular components by applying the whole preparation to the chromatographic origin has improved the reliability of the analyses.

The sulfolipid and the lysosulfolipid occur essentially only in the lamellar particulate fraction in *Lemma*. This result and other available data suggest that the sulfolipid is an integral part of the photosynthetic structure. It and the galactosyl dinolenins (10) are among the first lipids to have their biological function associated with their intracellular location. The sulfonic acid groups of the lipids are strongly anionic under all conditions; the surfactants properties of these amphiphatic molecules may be expected to stabilize active protein conformation and charge distribution in the quantum conversion apparatus. One can easily suspect that they thereby play a physical role in photosynthesis as well as being large reservoirs for reduced sulfur and carbohydrates (4, 7, 14, 22, 23). The remarkably different fatty acid composition of the sulfolipid (50% linolenic acid, 18:3 and 50% palmitic acid, 16:0) in alfalfa leaves (17) resembles that of typical phosphatides (lecithin) not present in lamellar lipoprotein. Spinach lamellar particles contained no detectable lecithin: this is a characteristic component of plant mitochondria and other membrane systems. Phosphatidyl glycerol is the major phospholipid of the lamellae. Its possible role in carbohydrate metabolism has been discussed (2).

Radiochemical analysis of the components in chloroplasts and in 3 chloroplast fractions from *Lemma*-C14 revealed that approximately 90% of the glycolipids were associated with the 110 to 145 P fractions. Since the 14 P, the chloroplast membrane fraction, was contaminated by considerable amounts of 110 P and 145 P components, it was unreasonable to suspect all the glycolipids were present only in the 110 P and 145 P fractions. These fractions, later simply designated as 145 P, were presumed to be equivalent, in size and chemical nature to the lamellar particles described by Park and Pon.

The results in Table I also reveal that the 2 galactolipids (cf. 10), galactosyl diglyceride and digalactosyl diglyceride, were concentrated in the quanta- somes whereas phospholipids and triglycerides were distributed in other fractions. The lamellar particulate preparations described here could have been contaminated by nonchloroplast components. It is, however, very unlikely that such contaminating fragments contained appreciable sulfolipid since it was absent in isolated mitochondria and ribosomes (22).

Radiochromatography of S15-Leuca perpusilla has revealed several other interesting features of this water plant. Sulfoquinovosyglycerol which has been found in high concentrations (22, 23) and shown unique metabolism (21) in green algae has not been detected in *Lemma*. An extremely high radioactivity, which was easily adsorbed on paper as seen in figure 1, was found in an unidentified spot with Rf values similar to those of green pigments. Although this lipid-like compound has also been de-

<table>
<thead>
<tr>
<th>Mole %</th>
<th>Mole %</th>
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<tbody>
<tr>
<td>Glycine</td>
<td>9.02</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.99</td>
</tr>
<tr>
<td>Valine</td>
<td>6.95</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.80</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.61</td>
</tr>
<tr>
<td>Proline</td>
<td>4.21</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.75</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.07</td>
</tr>
<tr>
<td>Serine</td>
<td>5.93</td>
</tr>
</tbody>
</table>
ected in S32-labeled green algae (21, 23), its cellular concentration has usually been much less than that of the sulfolipid. This compound was recently demonstrated to be associated with chloroplast development in Chlorella protothecoides and is now shown to be concentrated in the chloroplasts in *Lemna*. It may play an important role in sulfur metabolism and *Lemna* appears useful for the study of this compound.

The analyses shown in Table III indicate the typical lipoprotein-like nature of the lamellar particulate fraction from spinach leaves. A minor amount of nucleic acids found in the preparation indicates a minimal ribosomal content of chloroplast fragments (18). Both DNA and RNA should be present. Amino acid composition of the protein moiety shown in Table IV was not essentially different from those of average plant proteins, although somewhat higher contents in neutral amino acids and lower in basic amino acids could be pointed out. The result is similar to those reported by Menke (15) and Weber (25, 26).

These facts are in accord with the proposed hydrophobic association model of the lamellar lipoprotein (7, 27). They contraindicate a classical bimolecular lipid leaflet model for the lamellae which would require a protein of high basic amino acid content for stability of ionically bound lipid membranes. The striking difference between the fatty acid compositions of the 4 major surfactant lipids precludes nonspecific exchange of their hydrophilic moieties. The fatty acid complement of lipid hydrocarbon chains of the diglycerides probably decreases the site of their hydrophobic association within the lamellar lipoproteins, and thereby the location of the charged and hydrophilic groups at their exterior surfaces.

Summary

Radiochromatographic analysis of C14-labeled *Lemna perpusilla* revealed specific concentration of major glycolipids in the particulate fraction of sonically ruptured chloroplasts. Development of a procedure for quantitative isolation of the quantasomes directly from uniformly S32-labeled *Lemna* demonstrated that the sulfolipid and the lysosulfolipid are specifically localized in lamellar fragments or lipoprotein subunits of similar size. Lipid and amino acid composition of the spinach lamellar particulate fraction was examined. Their 4 major lipids are mono- and digalactosyl diglycerol, glyceryl, and sulfoquinovosyly diglyceride. This association in lamellar lipoprotein particles is discussed.

Acknowledgments

The authors are indebted to Dr. M. Miyano and Mr. C. S. Chen of the Institute of Applied Microbiology for their assistance in the cultivation of *Lemna* and to Mrs. S. G. Freer for analysis of spinach lamellar lipids.

Literature Cited

de- and re-generation of chloroplasts in the cells of *Chlorella protothecoides*. Plant Cell Physiol. 6: 267-83.


