The Flagellar Membrane of *Ochromonas danica*

**LIPID COMPOSITION***

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The lipids of the whole flagella and the flagellar membrane of the phytoflagellate *Ochromonas danica* were isolated and compared with those of the whole cell. The polar lipids were separated by two-dimensional thin layer chromatography. One-dimensional thin layer chromatography was used for the separation of the nonpolar lipids. In all respects the lipids of the whole flagella were identical with those of the flagellar membrane. These methods established the presence in flagellar membrane of the polychlorosulfolipids of *O. danica* as more than 90 molar per cent of the total polar lipids. These sulfolipids had been previously characterized as 1,14-docosanediol-1,14-disulfate and 1,15-tetracosanediol-1,15-disulfate, containing zero to six chloro groups substituting for hydrogen on the chain. Seven unknown polar lipids were found. Both phosphorus analysis on each lipid and the molybdenum spray reagent for phospholipids on the chromatogram showed that there is no phospholipid present in *O. danica* flagellar membrane. Positive reactions to the diphenylamine spray reagent suggest that up to four of the unknown polar lipids are glycolipids. All of the unknown lipids reacted with the acidified 2,4-dinitrophenylhydrazine spray reagent suggesting the presence of aldehyde, ketone, glycoside, or plasmalogen.

One unknown substance appeared near the origin of thin layer chromatograms. It showed a positive reaction with Dragendorff reagent, suggesting the presence of a quaternary amine group. This substance is presumed to be nonlipid, since it is not synthesized from [*1-C]acetate under the growth conditions used, as revealed by autoradiograms of thin layer chromatograms. It contained 35% hexose or hexosamine. It is devoid of phosphorus (0.07%) and is less than 4% protein (or phenolic groups or peptide), as judged by the Lowry assay using bovine serum albumin as a standard.

Analysis of the nonpolar lipids of the flagellar membrane showed that free fatty acids constitute about 12 molar per cent of the total lipids. These free fatty acids could be true components of the membrane or artifacts of the extraction procedure although every precaution was taken to prevent artifactual production of free fatty acids. The sterols constitute nearly 10 molar per cent of total lipids. Sterol esters were absent from the membrane. There are two additional major unknown nonpolar lipids present.

The implications of such a high proportion of chlorosulfolipids as a polar lipid component in the membrane are important because of the unique structures of these lipids, which have ionic groups at or near both ends of the aliphatic chain.

In the accompanying article (1) we describe the isolation and partial characterization of the flagellar membrane of the phytoflagellate, *Ochromonas danica*. The flagella were amputated by chilling and agitating the cell suspension briefly on a Vortex mixer. The removal of the flagellar membrane was achieved by simply suspending the whole flagella preparation in Tris buffer* at pH 7.5. A membrane preparation was obtained from the flagella by differential centrifugation and by sucrose density gradient centrifugation. The membrane preparation was deemed sufficiently pure for a lipid analysis, in addition to the electrophoretic analysis of the proteins previously conducted.

The lipids of *O. danica* are especially interesting because of the presence of a group of chlorosulfolipids which appear to dominate the polar lipid composition (2, 3). These substances are derivatives of 1,14-docosanediol-1,14-disulfate and of 1,15-tetracosanediol-1,15-disulfate with from zero to six chlorine substituents.

*The abbreviation used is: Tris buffer, 10 mM Tris-HCl, pH 7.5. 4 mM magnesium chloride, and 1 mM mercaptoethanol.

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atoms replacing hydrogen atoms on the saturated hydrocarbon chain (4-8). These lipids have polar groups (since they are charged at all physiological pH levels) at the proximal end of the chain, and also toward the distal end of the chain. Consequently, they do not fit a simple bilipid leaflet model. The presence of these substances as a major component of a membrane would therefore be of some interest.

This paper reports a detailed analysis of the lipids in the flagellar membrane of *O. danica* and compares it to that of the whole cell. The major component of the polar lipids is the mixture of chlorosulfolipids. The membrane is devoid of phospholipids.

**EXPERIMENTAL PROCEDURE**

*Ochromonas danica* was cultured in the dark in the chemically defined medium of Aarsonon and Baker (9) as previously described (1). Sodium [1-14C]acetate (4 mM) (specific activity: 1 mCi/mg) (New England Nuclear, Inc.) dissolved in ethanol, was added to a 3-day-old culture under reduced pressure. Cultures were maintained for 3 days in a dry box connected to three successive traps containing 1 M NaOH. The flagella and the flagellar membrane were isolated as described in the preceding paper (1).

Lipids were extracted with 20 volumes of chloroform/methanol, 2:1 (v/v) three times. The solvent was removed by rotary evaporation under reduced pressure. 2,6-Di-tert-butyl-4-methyl phenol (BHT) (0.01%, w/v) was added as an antioxidant. The whole extraction procedure was conducted under nitrogen.

**Chromatograph and Autoradiography**—Thin layer chromatography was conducted in two dimensions (for polar lipids) and in one dimension (for nonpolar lipids) using silica gel precoated plates. Plates were activated at 60°C for 1 hour and cooled in a desiccator. One-dimensional plates were developed with ether/hexane, 3:7 (v/v). Two-dimensional plates were developed in the first dimension with chloroform/methanol/28% (v/v) aqueous ammonia, 65:35:5 (v/v). Chromatograms were dried for about 10 min under nitrogen, placed in an oven at 40°C for 15 min, cooled in a desiccator, and then developed in the second dimension with chloroform/acetic acid/methanol/water, 5:2:1:0.5 (v/v/v/v/v) (10). Plates were obtained from two sources. Brinkmann Instruments (F-354) plates gave poor results on charring whereas better autoradiograms were obtained from these plates. Conversely, the chlorosulfolipids were chared with facility on Supelcoil 42A (Supelco, Inc., Bellefonte, Pa.) plates, although these plates were too powdery for autoradiogram preparation. These two types of plates yielded the best separations of the polar lipids.

The tightly capped tubes were placed in a boiling water bath for 2 min and cooled for 10 min, and the absorbance at 620 nm (Carl Zeiss M4QIII) was measured.

**Gas-Chromatography**—The fatty acid methyl esters were examined with a Perkin Elmer (801) gas chromatograph (flame ionization) and a 6-foot column of Gas-Chrom P (120 mesh) coated with diethylene glycol succinate (801) gas chromatograph (flame ionization) and a 6-foot column of Gas-Chrom P (120 mesh) coated with diethylene glycol succinate (12) using a Beckman LS-250 lithium chloride system.

**Hydrolysis of Chlorosulfolipids and Unknown 5**—In order to quantify the relative amounts of sulfolipid and Unknown 5, which overlapped on the thin layer chromatography plate during the 4C-labeled experiment (Figs. 1 and 5A) we hydrolyzed the mixture to free fatty acids and labeled diols (sulfolipid), which were subsequently separated and counted.

**Fatty Acid Composition of Free Fatty Acids**—The spot identified as free fatty acids on thin layer chromatography (Fig. 4) was scraped off the plate directly into a test tube. The silica was exhaustively extracted with acetone, and the combined extracts were evaporated under a stream of nitrogen. The residue was heated in a boiling water bath in 2 ml of methanol/boron trifluoride reagent (Applied Science). Water (2 ml) and ether (1 ml) were added to the solution. The ether was separated and combined with three subsequent ether washes. The ether solution was backwashed with water three times, dried with anhydrous sodium sulfate, and evaporated in vacuo. The entire procedure was conducted under nitrogen.

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** autoradiography—**Spots from Thin Layer Chromatography—Spots were scraped off the plates directly into test tubes and analyzed by the method of Rouser et al. (10).

**Protein Assay**—The concentration of protein was determined by the method of Lowry et al. (16), using bovine serum albumin as a standard.

**Azur A Colorimetric Assay for Sulfolipid**—The procedure of Kean (17) was used: samples were pipetted into screw-cap test tubes and evaporated to less than 0.1 ml. Each tube was added 5.0 ml of chloroform/methanol, 1:1 (v/v); 5.0 ml of 0.05 N H2SO4; and 1.0 ml of Azure A solution (40 mg in 5.0 ml of 0.05 N H2SO4, diluted to 100 ml with water). The tubes were capped, mixed for 30 s (Vortex), and centrifuged (300 × g for 5 min). The absorbance (Carl Zeiss MQIII) at 465 nm of the lower phase is a molar measurement using sodium dodecyl sulfate as a standard. It should be noted that the measurements of *O. danica* sulfolipids (with two sulfates on the molecule) must be halved to obtain molar quantities.

**Synthesis of Cholesteryl 17α-Dihydroxy-5α-Eicic acid (278 mg in benzene 20 ml) was cooled in an ice bath. Phosphorus trichloride (200 ml) was added, and the mixture was maintained in the ice bath for 1 hour. Cholesterol (389 mg) in benzene (10 ml) was added, and the reaction was left in the ice bath overnight. The solution was washed twice with saturated sodium bicarbonate, washed with water, and separated, and the benzene was dried with anhydrous sodium sulfate. After evaporation of the solvent, the cholesteryl oleate still had free oleic acid, which was useful in the chromatographic standard.

**Hydropysis of Chlorosulfolipids and Unknown 5**—In order to quantify the relative amounts of sulfolipid and Unknown 5, which overlapped on the thin layer chromatography plate during the 4C-labeled experiment (Figs. 1 and 5A) we hydrolyzed the mixture to free fatty acids and labeled diols (sulfolipid), which were subsequently separated and counted.

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response is greater for saturated acids). The error can be as large as 10%.

RESULTS

Lipid Composition of *Ochromonas danica* (Dark Grown)— Autoradiograms of thin layer chromatographic separations of *O. danica* lipids are shown in Fig. 1 and in Fig. 2. The polar lipids were separated by two-dimensional thin layer chromatography by Rouser's system (10) (Fig. 1). Phosphatidylcholine and phosphatidylethanolamine were identified by using Asolectin as a standard. Sterols and fatty acids were identified by co-chromatography with standards. Sphingosine-containing lipid had been found to be less than 0.3% of the total lipid, which was the limit of detection. Nonpolar lipids migrated to the upper left hand corner of the plate.

Fig. 2 shows a one-dimensional thin layer chromatogram of *O. danica* lipids developed in a solvent system of ether/hexane, 3/7 (v/v). The nonpolar lipids were separated, and the polar lipids remained at the origin. Cholesterol oleate was synthesized and used as a standard. Stigmasterol was also used as a standard. Six sterols have been identified in light grown *O. danica* (19), and all these sterols migrate with the same Rₜ values as stigmasterol. Comparison with standards indicates that there are free fatty acids and free sterols in *O. danica* lipids, but sterol esters were not detected. A chromatogram with an identical pattern was obtained by spotting the whole cell directly on thin layer chromatographic plates (5 μl, spotted once) without pre-extraction of the lipids with chloroform/methanol.

Polar Lipid Composition of *O. danica* Flagella and of Flagellar Membrane—The polar lipids of the flagella were separated by two-dimensional thin layer chromatography using Rouser's system. An autoradiogram of the lipids obtained from the flagella of [1-¹⁴C]acetate-labeled cells is shown in Fig. 3A. Fig. 3B shows the charred thin layer chromatogram of the polar lipids of the flagella separated by the same solvent system. The thin layer chromatogram of the flagellar membrane lipid was virtually identical with that of the whole flagella. The significant difference between the charred plate and autoradiogram is that the heavily charred Spot 1 is absent in the autoradiogram. This means that Unknown 1 is not synthesized using acetate as a precursor. The pattern of the flagellar lipids is simpler than the polar lipid pattern of the whole cell (Figs. 1 and 3A).

Preliminary Characterization of Flagellar Polar Lipids—Thin layer chromatograms of flagellar lipids co-spotted with Asolectin showed that the seven unknown polar lipids of the flagella do not co-chromatograph with phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, or phosphatidic acid. Both phosphorus analysis on each lipid and the molybdenum spray reagent for phospholipids on the chromatogram showed that the *O. danica* flagellar membrane contains no phospholipids. Preliminary characterization of the flagellar lipids (Table I) shows that Unknowns 1, 2, 3, 6, and 7 appear to contain glycoside. Unknowns 5 and 8, because they react only weakly with diphenylamine, do not appear to be glycolipids. Unknowns 1, 3, 6, and 7 contain nitrogen as judged by ninhydrin reaction. All unknowns reacted with acidified 2,4-dinitrophenylhydrazine, suggesting the presence of aldehyde, ketone, glycoside, or plasmalogen. One unknown substance, Unknown 1 (near the origin), appears not to be a lipid, as it was not labeled with [1-¹⁴C]acetate.

R. C. Gaver and T. H. Haines, unpublished experimental results.

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**Fig. 1.** Autoradiogram of a thin layer chromatogram of polar lipids from *Ochromonas danica* (dark grown). Crude lipid extract was spotted on the lower right-hand corner. Blue-sensitive no screen x-ray film was used. The adsorbent was Silica Gel F-254 precoated thin layer chromatography plates from Brinkmann Instruments Inc. Solvent System 1, chloroform/methanol/28% (w/v) aqueous ammonia, 65/35/5 (v/v/v); Solvent System 2, chloroform/acetic acid/water, 5/2/1/0.5 (v/v/v/v/v). The positions of known phosphatidylycerine (PC), and phosphatidylethanolamine (PE) are indicated.

**Fig. 2.** Autoradiogram of thin layer chromatogram of nonpolar lipids from *Ochromonas danica* (dark grown). Crude lipid extract was spotted on Silica Gel F-254 adsorbent. The solvent system is ether/hexane, 3/7. Blue-sensitive no screen x-ray film was used. Standards co-chromatographed with Unknown 11 (fatty acids) and Unknown 10 (sterols).
FIG. 3. A, autoradiogram of a thin layer chromatogram of polar lipids from Ochromonas danica flagella. The flagellar lipid extract was spotted on the lower right-hand corner. Blue-sensitive no screen x-ray film was used. Adsorbent, Silica Gel F-254 precoated thin layer chromatography plates. Solvent System 1, chloroform/methanol/28% aqueous ammonia, 65/35/5 (v/v/v); Solvent System 2, chloroform/acetone/methanol/glacial acetic acid/water, 5/2/1/1/0.5 (v/v/v/v/v). B, thin layer chromatography separation of polar lipids from O. danica flagella. Adsorbent, Supelcosil 42A precoated thin layer chromatography plates. Solvent, the same system as in A. Visualized by charring with 25% (w/v) sodium bisulfate containing 3% (w/v) H$_2$SO$_4$.

TABLE 1
Preliminary characterization of Ochromonas danica flagella polar lipids

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Xinhdyra (12, 13)</th>
<th>Diphenylamine (12)</th>
<th>2,4-Dinitrophenylhydrazine</th>
<th>Molybdenum trisulfide (12, 14)</th>
<th>Dragendorff (12, 15)</th>
<th>cis-Aconitate (15)</th>
<th>[1-14C]Acetate label</th>
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<tbody>
<tr>
<td>1</td>
<td>Unknown</td>
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<td>+</td>
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<td>+</td>
</tr>
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<td>-</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>4</td>
<td>Chlorosulfolipids</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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</table>

Numbers in parentheses refer to references.

Unknown 1—The spot near the origin (Unknown 1, Fig. 3B) was estimated by dry weight to be 5.8 times the quantity of sulfolipids. In order to establish this, the spot and the chlorosulfolipid spot were each scraped off the same thin layer chromatography plate (seven plates were run) and extracted with distilled water and redistilled chloroform/methanol, 2/1 (v/v), respectively. The chlorosulfolipids were assayed by the Azure A method. An average molecular weight of 615 was used. The unknown spot shows a strong positive reaction with Dragendorff reagent, suggesting the presence of a quaternary amine group. This same unknown spot is not synthesized from [1-14C]acetate (Fig. 3A), although the spot chars heavily (Fig. 3B). It shows a positive reaction with diphenylamine reagent. Carbohydrate was quantitatively assayed as 35% of its weight using the anthrone reagent. The spectrum of the anthrone adduct shows a major peak at 425 nm and a second broad peak at 640 nm identical with glucose. The implications of the former peak are not clear. These data suggest the absence of fatty acid in this lipid substance and the presence of carbohydrate. This unknown would constitute about 83% of the total flagellar lipid extract based on its weight ratio to the chlorosulfolipids if it had been included in the lipid composition. Its phosphorus content is less than 0.067% by weight. Its protein (or phenolic group and peptide) content is less than 4% by weight judged by the Lowry assay using bovine serum albumin as a standard. The spot also reacted positively with ninhydrin. It was not present in chromatograms of lipid extracts obtained from whole cells.
Nonpolar Lipid Composition of Flagella—The nonpolar lipids of the flagella were separated on thin layer chromatograms using ether/hexane, 3/7. An autoradiogram of the thin layer chromatogram shows essentially the same pattern as the thin layer chromatogram visualized by charring which is shown in Fig. 4. All of the spots shown are also exposed by iodine. Spot 10 has the same $R_f$ value as sterol. Spot 11 has the same $R_f$ value as fatty acid. Spots 9 and 12 neither absorb at 254 nm nor fluoresce at 366 nm. Spots 11 and 13 fluoresce at 366 nm. Spot 10 absorbs at 254 nm and fluoresces at 366 nm, entirely consistent with its assignment as the sterols of *O. danica* (19).

Quantitative Analysis of Lipids in Flagella and in Cells—Sodium $^{14}$C-lactate was added to a culture of cells in the log phase of growth, and the culture was maintained for 3 days. The label was used as a specific quantitative assay of the cellular lipids. The complete absence of label in the Unknown 1 spot supports this contention, as 35% of this spot has been shown to be carbohydrate.

The specific activities of the three major components of the lipid extract were determined directly. The lipids of deflagellated cells were extracted using the Folch procedure (20), and the lower layer was separated on one-dimensional thin layer chromatograms. The fatty acids and sterols were isolated, weighed, and counted. The "average" molecular weight used for these three constituents of the lipid extract was obtained by averaging the quantities of the individual components of known molecular weights. The relative quantities were determined by gas-liquid chromatography. The specific activities of the fatty acids ($1.5 \times 10^9$ cpm/mmol), the sterols ($1.8 \times 10^9$ cpm/mmol), and the chlorosulfolipids ($0.2 \times 10^9$ cpm/mmol) were based on average molecular weights of 276, 407, and 457 (diols), respectively. Unknown lipids were averaged as having two stearate chains based on the fatty acid composition of the cells (21), or of the flagella as measured. Using this approach, the lipid composition is described in Table II.

Unknown 5 did not separate cleanly from the chlorosulfolipid mixture. The mixture was scraped from the plate, extracted from the silica, hydrolyzed in 1 N hydrochloric acid, and rechromatographed. The fatty acids separated from the diol mixture, and were counted separately to obtain the relative amounts of each. This experiment shows that Unknown 5 contains hydroxyalkyl fatty acid esters.

Free Fatty Acid Composition of Flagella—The free fatty acids were scraped from a thin layer chromatography plate, extracted from the silica gel three times with about 10 volumes of chloroform/methanol, 2/1 (v/v). The solvent was removed under nitrogen, in vacuo. The fatty acids were converted to the methyl esters by the use of boron trifluoride in methanol. The methyl esters were separated on a 10% diethylene glycol succinate column on Gas-Chrom Q. The results of the analysis are shown in Table III.

Lipids to Protein Ratio—The lipid to protein ratios of the cells, the flagella, and the flagellar membrane are shown in Table IV. The low value in the whole flagella is undoubtedly due to the axonomes and mastigonemes, which are polymeric protein strands.

**DISCUSSION**

Flagellar Membrane Composition—The membrane preparation from the flagella of *O. danica* has been examined for three major constituents; protein, lipid, and Unknown 1, which is 35% carbohydrate. The lipid to protein ratio (Table IV) is 1.3, and the lipid to Unknown 1 ratio is 0.2. Nearly all of the lipids of the membrane appear to be unique. A prominent exception is the sterols, which appear to be typical algal sterols (19). There are no phospholipids in the membrane. The dominant lipids, in addition to the sterols, appear to be a mixture of algal sterols—derivatives of 1,14-docosanediol-1,14-disulfate (4, 5) with up to six chloro groups replacing hydrogens on the chain (2, 6-8, 22). and free fatty acids. We know of no membrane which contains prominent amounts of free fatty acids. The fatty acids could conceivably be artifacts produced during the extraction, although every attempt was made to minimize the possibility. The chlorosulfolipids, however, cannot be considered an artifact.

Quantitative Analysis of Lipid Composition—The lipid composition can be analyzed quantitatively by dry weight, or by radioactivity using $^{14}$C-labeled precursors of lipids. When unknown lipids are present the dry weight does not give the molar ratio of the lipids. Radioactive labeling with 1-14C-lactate would give a molar response, provided that it did not label the polar group of unknown dicylglycerides and that the fatty acid composition of the sample was known. Additionally the sterols and other nonpolar lipids are labeled in predictable ways depending upon the structure of the lipid.

The use of this approach for this system is based upon the composition of the chemically defined culture medium, which includes several amino acids and a large supply of glucose. It
**Ochromonas Flagellar Lipids**

**TABLE II**

*Analysis of lipids in Ochromonas danica flagella and whole cells*

\[ ^{13}C\]acetate was incubated with the cells for 3 days (eight cell divisions) in a chemically defined medium (see under "Experimental Procedure") containing amino acids and glucose. Lipids were separated on thin layer chromatograms and counted in a scintillation counter after being scraped off the plate. The radioactivity was considered to be in the fatty acid portion of each unknown lipid molecule. Molar per cent of an unknown refers to the fraction of that lipid present based upon a ratio of two stearic acid chains assumed to be in each unknown. The molar specific activity of the fatty acids, the sterols, and the chlorosulfolipids (as diols) was determined directly. Unknown 1, which is 30% carbohydrate (anthrone), contained no label from \(^{13}C\)acetate. It was estimated by its weight in milligrams to be 5.8 times the weight of the chlorosulfolipids.

<table>
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<td>12.3</td>
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<td>4.2</td>
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<td>0.2</td>
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<tr>
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<td>Unidentified polar lipids in cells</td>
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* a PC, phosphatidylethanolamine; PE, phosphatidylserine.

**TABLE III**

*Free fatty acids of Ochromonas danica flagella*

Values are expressed as weight percentage of composition based on a gas chromatogram of the methyl esters on diethylene glycol succinate (10% on Gas-Chrom P). The unsaturated esters include: palmitoleate (C<sub>16:1</sub>), olate (C<sub>18:1</sub>), linoleate (C<sub>18:2</sub>), γ-linolenate (C<sub>18:3</sub>), and arachidonate (C<sub>20:4</sub>). Traces (Tr) of α-linolenate and 13 other esters were observed. Because a flame ionization detector was used, the polyunsaturated acids are present in relatively higher amounts and the saturated fatty acids in relatively lower amounts than indicated.

**TABLE IV**

*Lipid to protein ratio*

The lipid analyses are based on radioassay of the lipid extract of cells cultured in the presence of \(^{13}C\)acetate. The specific activity of the isolated lipids was found to be \(8.0 \times 10^3\) cpm/mg of lipid. It was assumed that the specific activity was the same in each fraction, although the determination of the specific activity was made on the whole cell lipid extract. The protein was estimated with the Lowry method (16) using bovine serum albumin as a standard.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Lipid/protein (mg/mg)</th>
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<tbody>
<tr>
<td>Whole cells (dark grown)</td>
<td>1.1</td>
</tr>
<tr>
<td>Flagella (whole, including axonemes)</td>
<td>0.46</td>
</tr>
<tr>
<td>Flagellar membrane</td>
<td>1.3</td>
</tr>
</tbody>
</table>

was felt that the carbohydrate and protein of the cells would be poorly labeled from \(^{13}C\)acetate at best. This was confirmed experimentally by the absence of radioactivity in Unknown 1, which contains 35% carbohydrate.

This method appears, therefore, to be useful and relatively simple for establishing the molar ratios of the lipids. In order to calculate these ratios some assumptions are made with respect to the structures of the unknowns. An analysis of the fatty acids of *O. danica* shows an average chain length of just under 18 carbons. The six unknowns, which amount altogether to 9% of the total lipids based on raw counts, were considered to be diacylglycerides with average fatty acid chains of 18 carbons. The chlorosulfolipids were considered to have 11 acetates incorporated into each molecule (20, 23), although some tetraacetylsulfates are undoubtedly present (2). The sterols are considered to have 12 carbonyl carbons incorporated into the molecule, based upon their structure (19) and the general biosynthetic route of sterols. The alkyl side chains of the plant sterols are derived from methionine (24).

The specific activities of sterols and free fatty acids differ by about 12%, calculated on a per \(^{13}C\) basis using the above biosynthetic schemes. In contrast, the chlorosulfolipids have one-eighth of the specific activity of these two. The slow labeling of the chlorosulfolipids is entirely consistent with the slow biosynthesis of these substances as observed by Thomas and Mercer (25). The similarity of the specific activities of the fatty acids and of the sterols suggests that where the source of the label in an unknown is primarily fatty acids or mevalonate the results are comparable. The estimates of relative molar amounts in Table II are based on the specific activities of these three compounds.
**Polar Lipid Composition of Flagellar Membrane**—The lipid composition of the flagellar membrane and that of whole flagella were found to be qualitatively identical by charring and spray reagent patterns of two-dimensional thin layer chromatograms. The results reported here refer to analyses of whole flagella. The chromatograms are shown in Fig. 3. The list of polar lipid components of those chromatograms can be found in Table II. Table 1 shows their reactions with a variety of specific reagents.

Probably the most outstanding facts about the membrane polar lipid composition are the total absence of phospholipids and the presence of a dominant fraction (90 molar per cent of polar lipids) of chlorosulfolipids. The former, although surprising, is not as surprising as the latter. The absence of phospholipids does not have important implications to the membrane structure as they may be replaced by other ionic or polar lipids with analogous structure. This has already been noted in *O. danica* by Elovson who has identified 1(3),2-diacylglycerol-3(1)-O-4’-(N,N,N-trimethyl)homoserine (trimethylhomoserine diacylglyceryl ether) as one of the major lipids in the photoflagellate with steric and ionic-hydrophobic structural properties similar to those of the phospholipids (26). One may presume the Unknowns 2, 3, and 5 to 9 are analogous in their steric and ionic-hydrophobic structures to the phospholipids. These compounds, however, constitute together only 8.7 molar per cent of the total polar lipids in the flagellar membrane. This is a maximum, since the structures of these compounds are unknown. To our knowledge, this is the first report of a membrane totally devoid of phospholipids. This fact has a significance of its own, since the phospholipids are generally considered critical components of biological membranes (27, 31).

Another aspect of the analysis of the flagellar membrane polar lipids that emerges as an important finding is the presence of a dominant fraction of chlorosulfolipids. These lipids are unsuitable for forming bilayer membranes as (a) they are very water soluble; (b) they would have a second ionic group too deep in the hydrophobic region of the bilayer for stability; and (c) they could not “loop” back so as to have both groups on the polar surface of the bilayer, as the chain would only penetrate about eight carbons into the hydrophobic layer. These factors make a bilayer of the chlorosulfolipids possible only if there are positive charges deep in the hydrophobic region to shield the negative sulfate groups. (Sulfate must be charged at any physiological pH, since the pK is below 1.) Only two such cations come to mind—one is divalent metals, which would bridge two such groups, and the other is hydrophobic protein or other macromolecule which penetrates the bilayer. It is possible to distinguish between these, and studies are planned to make this distinction.

Several laboratories have noted the continuity of flagellar or cilia membranes and the cell membrane. The electron micrographs obtained by us are in complete agreement with this observation. Additionally, freeze fractures of *O. danica* cell membrane show the membrane has a fracture plane typical of bilayers (3). It would appear, therefore, that the awkward structure of the chlorosulfolipids is somehow compensated in the bilayer by other membrane components.

Brown and Elovson (28) who characterized trimethylhomoserine diacylglycerol ether in *O. danica* have suggested that this substance, which constituted over 50% of the “normal” polar lipids, “rather than the detergent-like chlorosulfolipids, largely substitutes for the usual phospholipids in membrane bilayer structures in *O. danica.*” This suggestion is based on the disturbing prospect of imagining a bilayer with these alkyl disulfates as dominant lipids. The data presented herein lead to the inevitable conclusion that analogs of the phospholipids are not dominant in this membrane. The large quantity of these chlorosulfolipids (91% of the polar lipids) leaves little doubt that these substances dominate the polar lipid composition on both sides of the membrane bilayer. In view of the fact that these substances are potent detergents and denature enzymes even in relatively dilute extracts (6), it is remarkable that both the inside and the outside of the flagellar membrane would contain large amounts of a substance that is very water-soluble.

Of the unknown polar lipids, Unknown 8 dominates the flagellar membrane composition. This is the only unknown polar lipid that does not react with diphenylamine. The others are apparently glycolipids. This suggests a variety of unusual glycolipids in *O. danica.* It should be noted that *O. danica* could only contain less than 0.3% of its polar lipids as sphingosines, since that was the limit of detection used in an analysis of the long chain bases.

**Nonpolar Lipid Composition of Flagellar Membrane**—The nonpolar lipids of *O. danica* flagellar membrane have four major components. The bulk of the nonpolar lipids consists of the sterols and free fatty acids. The latter is discussed in the next section. Two unknown classes (Unknowns 12 and 13) constitute less than 2 molar per cent of the nonpolar lipids. The nature of these substances remains unknown. They are not sterol esters as they do not co-chromatograph with cholesteryl oleate. The sterols were previously characterized in this laboratory as ergosterol, brassicaster, 22-dihydrobrassicaster, clionasterol, poriferasterol, and 7-dehydroporiferasterol (19). The relative composition of the sterols is approximately that reported for the whole cell. It is interesting to note that the moles per cent of sterol are in excess of that for all of the polar lipids taken together—excluding the chlorosulfolipids. This is in agreement with many reports of widely variant polar lipids to sterol ratios (32).

**Free Fatty Acids**—Free fatty acids represent 12.3 molar per cent of the total lipids in the flagellar membrane. The occurrence of free fatty acids can be explained in at least three ways. They could be present in the flagellar membrane, or be produced by a very active lipase in the lipid extract, or result from a labile hydrolysis. *O. danica* grows in acidic media from pH 4 to pH 5. Cells do not grow at pH levels much higher than 5. More than 50% of the free fatty acids become charged at pH levels higher than 4.5, which is, interestingly enough, coincidental with the pH range of growth of *O. danica.* It is conceivable, although unlikely, that the bulk of uncharged free fatty acids fit in the membrane in the acidic pH range (pH 4 to 5), and this may offer one explanation to the inability of *O. danica* to grow at neutral or higher pH, which is optimal for many other organisms. On the other hand, active phospholipase and galactosidases of the photosynthetic tissues of plants have been reported in lipid extracts, and the addition of boiling isopropanol has been employed to inactivate the enzymes prior to extraction by chloroform/methanol (1/1, v/v) which appears to activate lipolytic enzymes (33). It is also conceivable that for defensive or nutritive purposes *O. danica* has a very high lipase activity. Attempts to inhibit lipase activity with boiling isopropyl alcohol did not substantially change the lipid composition in our studies. An attempt to avoid the lipase problem was also made by spotting the water suspension of whole
flagella and whole cells on thin layer chromatography plates before treatment with any organic solvent. Under these circumstances it is presumed that the lipase would have less opportunity to attack lipids. The developing solvent serves as an extracting solvent as well. This method is especially applicable when highly radioactive material is available, as only a small amount of material is extracted by the developing solvent and a highly sensitive visualizing method is available. The autoradiograms showed that the relative quantity of free fatty acid does not change using this procedure. This experiment argues against lipase activity during extraction as an explanation for the large quantity of free fatty acids we obtained in the membrane preparation.

Unknown 1—Fig. 3B shows that near the origin there is a heavily charred spot, not completely separated from the origin. This spot, Unknown 1, is seen only in the flagellar lipid extracts, not in the lipid extract of the whole cell. Quantitative anthrone analysis of this unknown isolated from thin layer chromatography plates showed it contained 35% carbohydrate. The spectrum of the anthrone product showed only hexose. Neither pentose nor deoxy sugars were present (34, 36). Our results indicate the presence of amino acids or amino sugars and quaternary amino groups. Phosphate and protein were essentially absent. The substance dominates the lipid extract by weight (83%, of the total extract) and yet is not labeled from [1-14C]acetate. The nature of this material and its function remain unknown.

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