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The City University of New York, Ph.D., 1974
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ISOLATION AND CHARACTERIZATION OF THE PLASMA AND EXTRACELLULAR MEMBRANES OF *OCHROMONAS DANICA*

BY

EDWIN W. BILLMIRE, Jr.

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1974

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

July 30, 1974
Date

Chairman of Examining Committee
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August 13, 1974
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ABSTRACT

A procedure developed for the isolation of the plasma membrane of Ochromonas danica is reported here. This membrane is compared to a vesicle fraction isolated from the secretions of this organism. The homogeneity of these membranes was judged by chemical, enzymatic marker, morphological and centrifugal criteria. The yield of plasma membrane was 13.4% of the cell dry weight while the extracellular membrane component, ECM-2, which was similar to the plasma membrane in chemical composition, comprised 79% of the total extracellular membrane (ECM). The yield of ECM-2 as % of cell dry weight was 5.8% of the cell dry weight.

The lipid to protein ratio of the plasma membrane and ECM-2 were 0.979 and 0.991 respectively by weight and the density of both membranes was estimated to be between 1.18-1.20g/ml sucrose. Lipids were examined from the whole cell and the membrane and organelle fractions. A fraction of the total lipid that was eluted from a silicic acid column by acetone was found to be concentrated in the plasma membrane and the ECM-2. Also enriched in these fractions were the sulfatides which accounted for 45% of the membrane lipid; this was a more than a two-fold increase over the sulfatide found in the whole cell and much greater than that found in other subcellular fractions.

Approximately 50% of the total phospholipid in the plasma membrane and ECM-2 is phosphatidyl choline while phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl glycerol were found to be equally distributed among the phospholipids of the whole cell.

The plasma membrane was enriched 4-16 fold in the specific activity of the following enzymes; B-galactosidase, acid phosphatase, alkaline phosphatase, 5'-nucleotidase and Na^+,K^+ ATPase. There was a 3-12 fold enrichment in B-galactosidase and 5'-nucleotidase in the extracellular membrane fraction.

There appeared to be a morphological and biochemical continuity between the plasma membrane of Ochromonas and part of the extracellular membrane which it produced. The biological implications of this relationship are discussed.

Acknowledgments:

I wish to thank Professors Aaronson and Haines for their advice and guidance in this work; Dr. N. Patni for his technical assistance and portions of the enzyme data; Audrey Donellan for her clerical assistance; and a special kind of thanks to my wife Pat and our children for their encouragement and devotion during the preparation of this thesis. This work was supported in part by a grant, GB 2082, from the NSF to S. Aaronson.

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INTRODUCTION

Ochromonas danica, a fresh water phytoflagellate, offers an attractive model for the study of biological membranes. There was ultrastructural, (Aaronson et al, 1971), centrifugal and spectrophotometric (Aaronson, 1971) evidence for the production of a wide variety of intra and extracellular vesicles and myelin-like bodies by Ochromonas. The extracellular membrane (ECM) had the trilamellar appearance of other biological membrane and appeared at the cell surface and in the cell-free growth medium from which it was collected by centrifugation (Aaronson, 1971). The ECM was shown to be a heterogeneous collection of membrane of several origins.

One possible site inferred from electron micrographs of O. danica for the formation of the ECM was the plasma membrane which appeared to have protrusions and accumulations of vesicles at and escaping from its cell surface. Another possible site was the chloroplast membranes which appeared to give rise to myelin-like bodies described as extending from the chloroplast surface until they broke away into the cytoplasm and then out of the cell (Aaronson, 1971). The above two membranes have distinct morphological and molecular characteristics. In animal, plant and some eucaryotic microorganisms subcellular membranes were studied by a comparison of their

morphological, centrifugal, chemical and enzymic properties. Intracellular membranes and vesicles have been separated according to density by isopycnic centrifugation (de Duve, 1971). The ECM was therefore separated into more homogeneous fractions by differential and isopycnic centrifugation and characterized according to the criteria utilized for intracellular membrane (Benedetti and Emmelot, 1968).

The subcellular organelles and plasma membrane of O. danica were separated and characterized so that a comparison could be made between them and the ECM subfractions. Since morphological evidence pointed toward the plasma membrane as a possible source for ECM, emphasis was placed on obtaining a purified plasma membrane preparation. The plasma membrane of very few eucaryotic microorganisms have been isolated. Among these few plasma membranes were: ameba (Schultz and Thompson, 1969; Korn and Olivecrona, 1971), yeast (Schibece et al, 1973) and a trypanosomatid flagellate (Hunt and Ellar, 1974) but none from algae, or phytoflagellates. A method for the isolation and purification of the plasma membrane of Ochromonas danica was developed and is described in the work reported here. Some of the criteria used for determining the identity and purity of plasma membrane came from the extensive studies on the biological membranes of mammalian cells (Benedetti and Emmelot, 1968), and include 1) microscopy; 2) centrifugal

patterns; 3) chemical composition; and 4) enzymic markers. These criteria were used to identify the plasma membrane, other subcellular organelle fractions, and the ECM. The several organelle and membrane fractions can be distinguished by their microscopic and ultrastructural appearance. Although electron microscopy is indispensable toward defining membrane homogeneity, this technique was augmented by assaying enzyme activities and chemical composition for molecules known to be associated with the plasma membrane and other cell fractions of cells whose membrane composition had been defined.

The lipids of Ochromonas were of special interest as potential chemical markers for characterizing the intra- and extracellular membranes developed by the fractionation procedure because: 1) The centrifugate collected from the cell-free medium was reported to contain a high proportion of lipid which made up 76% of its dry wt. (Aaronson, 1971); 2) Studies detailing the lipid composition of subcellular membranes led Fleisher (1967) to suggest that each membrane bound organelle had a characteristic lipid profile or specific marker lipid molecule. Cardiolipin is a good marker for mitochondria. Galactolipids have been found in plant and algal chloroplast membranes in large quantities (Allen et al, 1965). There is a marked difference in the sterol content of surface and internal membranes of animal cells (Coleman and Finean, 1966; Coleman, 1968); 3) A specific group of polar

lipid, the sulfatides, were reported by Haines and Block (1962) to be excreted into the growth medium of O. danica. When the centrifugate from the Ochromonas cell-free medium was examined, it contained large amounts of sulfatide (Gellerman and Schlenk, 1964). Kahn and Haines (Haines, 1973) demonstrated that sulfatides appeared in a peak in a Ficoll density gradient that was designed for the examination of membrane by Kamat and Wallace (1965); 4) Specific glycolipids were found in the plasma membrane of animal cells (Weinstein et al, 1970). Nichols and Appleby (1969) reported the presence of two unidentified lipids called A and B in Ochromonas, chromatographically similar to cerebrosides and ceramides which are components of animal cell plasma membrane. Phytocerebrosides were found in lipid extracts of plants, however their location was not established (Schwertner and Biale, 1973).

A more extensive lipid analysis of O. danica was needed in order to utilize lipids as markers for membrane characterization. A wide variety of lipids had been found in this organism. The fatty acid (Haines et al, 1962); Nichols and Appleby, 1969), sterol (Gershengorn et al, 1968), and sulfatide (Haines, 1973) composition had been quantiated. Nichols and Appleby (1969) identified the major phospholipids and glycolipids. Chlorophyll (Allen et al, 1960) and B-carotene (Allen et al, 1960a) were shown to be present. Quantitative data on the

individual lipid classes and the phospholipid classes was not available; therefore, such analyses were the initial undertaking of this work followed by similar analysis of the several fractions once identity and homogeneity had been established.

The several fractions could be compared when the morphological and molecular profiles were determined. Molecular markers which define homogeneity of a specific membrane or organelle may also suggest a continuity between two membrane types such as the plasma membrane and a fraction of ECM. If there is a morphological and biochemical continuity between a specific cytomembrane and extracellular membrane, this continuity may suggest the development of the ECM from the cellular membrane. The reverse is known to occur during pinocytosis or phagocytosis. Ulsamer et al (1971) have demonstrated such a continuity between the phagosomes and the plasma membrane of ameba.

The biological role of excreted or secreted membrane is not clear. Extracellular membranous structures also have been reported in mammalian cells (Sun, 1966), in slime mold (Mercer and Shaffer, 1960) and other phagocytic cells (Aaronson et al, 1971; Vickerman, 1962). Several possible roles for O. danica ECM were examined. Among these were 1) the agglutination or immobilization of other microbial cells (Aaronson, 1971a); 2) the aggregation of small

particles at the cell surface; and 3) association of 10% of the extracellular hydrolases found in this organism with the ECM (Shio, 1971). One may infer a biological role for the ECM from such observations, in which the ECM serves to trap and engulf particles and food organisms from its environment. Similar observations have been made in other microorganisms; certain species of Dinoflagellida, Euglenida and Cryptomonadida produce and extrude trichocysts that paralyze prey which they then feed upon (Aaronson, 1973a); euglenoid flagellates (Leedale, 1967) and at least one chrysophyte Prymnesium parvum (Manton and Leedale, 1967) extrude muciferous bodies from their surface which may serve to trap food.

Methods

The organism and growth condition

Ochromonas danica strain L933/2 Pringsheim was maintained in a chemically defined medium (Aaronson and Baker, 1959) in a 5 ml volume in screw-cap test tubes in a refrigerated incubator at 25°C with 11.6-14.0 lux of white fluorescent light. Experimental cultures were grown in the same medium under the same conditions in 500 ml or 2000 ml volumes in one liter screw-cap Erlenmeher or 2.8 liter Fernbach flasks respectively. Flasks were incubated with 20,000 cells per ml from a 48-72 hr. culture and incubated for 5 days (log/ phase).

Harvesting and preparation of homogenate

All centrifugation procedures were carried out at 4°C. Log phase cells were harvested by centrifugation at 4,340 x g for 10 min for the purpose of collecting whole cells and cell-free medium, and at 1,000 Xg for 10 min for the purpose of collecting cells to be used for fractionation. Centrifugation of cells was carried out in a Sorvall RC-2B refrigated centrifuge. The cell-free medium was poured off, examined with a phase contrast microscope for cells. The cell-free medium was further examined

either for lipid material or recentrifuged first at 41,000 X g for 30 min to remove cell-debris (large vesicles and microtubules). The supernatant from this centrifugation was removed and recentrifuged at 105,000 X g for 60 min in a Beckman preparative ultracentrifuge Model L3-40 with a Ti 50 rotor to recover the extracellular membrane material (ECM). The ECM was further purified; this will be discussed later in Methods. Dry weights of all samples were determined in triplicate on aliquots dried at constant weight at 60°C. If not analysed or extracted for lipids immediately, the samples were stored at 20°C.

Cells to be fractionated were suspended in homogenization buffer. The homogenization buffer was a modification of the Eisenstadt and Brawerman medium (1964). The composition of the buffer was 0.25M sucrose, 0.01M Tris-HCl buffer (pH 7.6), 0.5mM $MgCl_2$ and 1.0 mM B-mercaptoethanol was added to the buffer each day prior to use. This solution was designated STM. Cells from 500 ml cultures were suspended in 40 ml of STM buffer. The cell suspensions were treated in a cold French Pressure cell (Aminco, Silver Springs, Maryland) at 3.5-619 X 106 Nm^{-2}. This treatment brought about a nearly quantitative cell disruption.

Fractionation

The resulting cell homogenate was centrifuged in a Sorvall RC2-B at 480 X g for 10 min to remove nuclei,

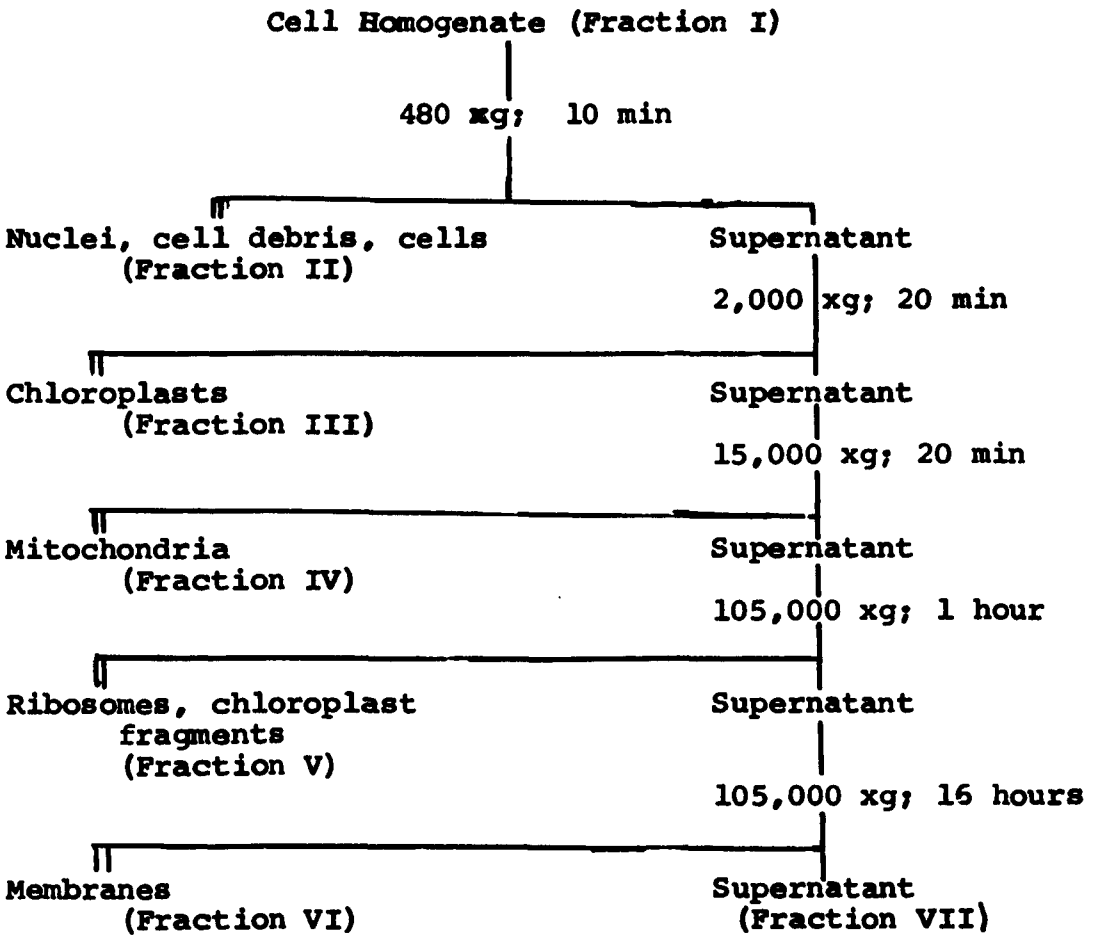
broken cells and the few whole cells that remained unbroken. The supernatant was centrifuged at 2000 X g for 20 min to remove the chloroplast fraction and again at 15,000 X g for 20 min to remove the mitochondrial fraction. Supernatant from the last centrifugation was centrifuged at 105,000 X g for one hour in the ultracentrifuge with the Ti50 rotor to remove the ribosomes, organelle membrane fragments and the supernatant again centrifuged for 16 hr. at 105,000 X g.

The membrane Fraction VI was further purified by being dispersed in 2ml of 10% sucrose-STM buffer and then treated in a manner similar to that of Bosmann et al (1968). The dispersed Fraction VI was layered on the following discontinuous sucrose density gradient: sucrose concentration from the bottom to the top of the tube were 1ml of 75%, 19ml of 45%, 13ml of 35%, 13ml of 30%, 10ml of 25%. The gradient was centrifuged at 76,000 X g for 16 hrs in the L3-40 ultracentrifuge in an SW25-2 rotor. Four distinct bands were resolved and fractions were collected by taking up the fraction with a syringe. The fractions from the top to bottom were designated VI A, VI B, VI C, and VI D. This fractionation procedure is summarized in figure 1.

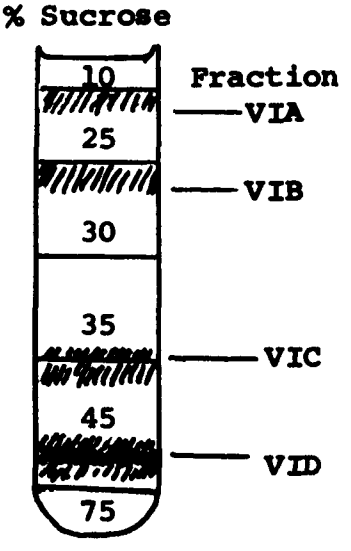
Purification of the ECM

Cell debris was removed by centrifugation of the cell-free medium at 41,000 x g for 30 min. The ECM then was collected as a pellet at 105,000 X g after 60 min of

Figure 1. Flow chart of fractionation of O. danica cell homogenate



An aliquot of Fraction VI was placed on a discontinuous sucrose density gradient and resolved into 4 fractions after centrifugation at 76,000 X g for 16 hours.



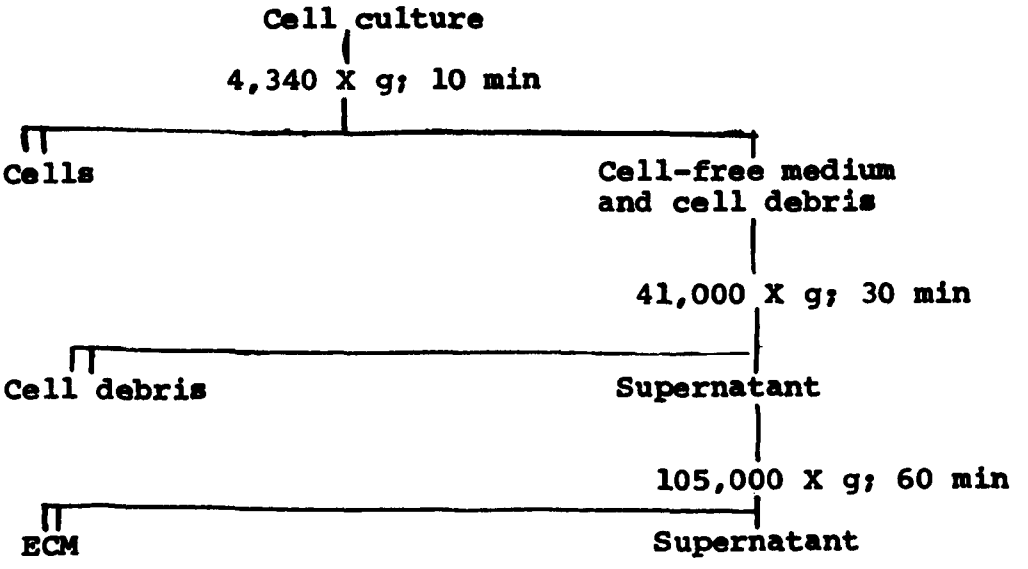
centrifugation in the Beckman ultracentrifuge. This material was either analysed directly or resuspended in 2ml of STM and layered on a discontinuous ducrose density gradient the specifications of which was described in the previous section. This gradient was centrifuged for 16 hrs at 76,000 X g in an SW25-2 rotor. The ECM was resolved into two membrane bands designated ECM-1 and ECM-2 and collected from the gradient with a syringe. The collection and fractionation of the ECM is summarized in Figure 2. The collected material was washed by diluting 10:1 with sucrose-free STM and recentrifuging for one hour at 105,000 X g to pellet the membrane. The membrane was either analysed directly or stored at -20°C.

Lipid analysis

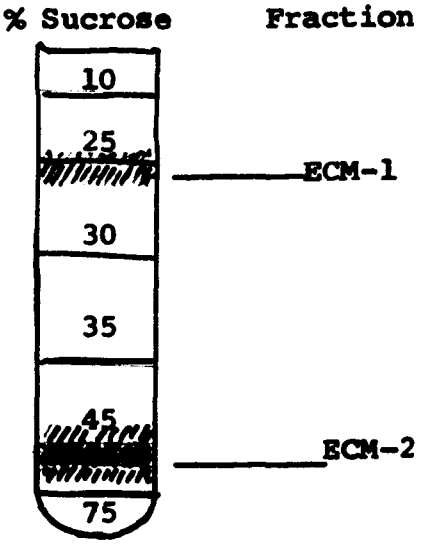
Extraction

All material to be extracted was either fresh or had been kept frozen at -20°C. All solvents were reagent grade, redistilled and bubbled with nitrogen prior to use. Lipid extraction and analysis was performed under an atmosphere of nitrogen to minimize oxidation of poly-unsaturated fatty acids. An antioxidant, 2,6-di-tert-butyl-4-methyiphenol, (Aldrich), and the hydrolytic enzyme inhibitor 5,5'-dithiobis-(2-nitrobenzoic acid) (Aldrich), were added to the lipid extracts to avoid oxidation and degradation of lipid material (Ray et al, 1969). The cell material was extracted with 19 volumes

Figure 2. Flow chart for the collection and fractionation of the extracellular membrane (ECM).



An aliquot of the ECM was layered onto a discontinuous sucrose density gradient and resolved in 2 fractions after centrifugation at 76,000 X g for 16 hours.



of chloroform-methanol (2;1, v/v). After standing at room temperature for 3 hrs all the extraction mixtures were filtered and the filtrate was reextracted with chloroform-methanol. This was repeated a third time and the extracts were combined and partitioned into upper and lower layers in a separatory funnel with 0.2 volumes of distilled water according to Folch et al (1957). Lower and upper layers were washed twice with .05 volumes of upper and lower solvent phases respectively. The wash was added to the appropriate phase and the respective phase concentrated *in vacuo*. The lower chloroform phase contained all of the neutral and most of the polar lipids and was never taken to dryness *in vacuo*. After volume reduction the lipids were removed by replacing the vacuum with nitrogen and the residue was redissolved in a small volume of chloroform-methanol (2;1, v/v). The upper methanol-water phase contained the polar sulfatides and was redissolved in a small amount of butanol. The butanol containing the upper phase lipid, salts and some protein was then filtered through S&S No. 507 filter paper to remove salts and protein and analysed for lipid.

When cell-free medium was extracted directly for lipids two modifications of the above procedure were made. First the volume of medium was reduced *in vacuo* from 2 liters to 50g wet weight at 10°C and in the presence of antioxidant and enzyme inhibitor. Secondly, this

material was extracted with 19 vol. of chloroform-methanol (2;1, v/v) by adding the methanol first to loosen the residue and then adding the appropriate volume of chloroform and mixing. All other procedures concerning the cell-free medium were carried out as described above for the cell material.

Fractionation of the lipid material by column chromatography

Lipid extracts were fractionated into their respective bulk lipid classes on a silicic acid column by a modification of Rouser et al (1967) procedure. The column was prepared by adding a slurry containing 15g of silicic acid G, mesh size 100-200 in chloroform into a 20 cm long glass column 2.5cm in diameter to a height of 5cm. Three column volumes (3 X 18 ml) of chloroform were allowed to pass through the column which had been fitted with scintered glass filter, a teflon stopcock, and a piece of glass wool to retain the absorbant. The solvent level was allowed to descend to the top of the silicic acid bed and 50 to 200 mg of lipid in 5-10 ml of chloroform or butanol were applied to the solvent above the silicic acid bed. The lower phase lipids were added first and the following solvents were allowed to percolate through the column under a head of nitrogen at a flow rate of 3ml/ min. Neutral lipids were eluted first with 10 column volumes of

chloroform (about 175 ml). The more polar or complex lipids were then eulted as follows: glycolipid (MGDG) with 5 column volumes (90 ml) of chloroform-acetone 1;1, (v/v), a modification introduced by Vorbeck and Marinetti (1965); glycolipids (DGDG, SQDG, unknown lipids A and B) with about 700 ml or 40 column volumes of acetone; the phospholipids with 10 column volumes (175 ml) of methanol; The upper phase lipids were then added in butanol and the sulfatides were eluted with 10 column volumes or 175 ml of butanol. The eluates were reduced in vacuo and analysed directly, further fractionated, or stored at -20°C under nitrogen.

The neutral lipids were further factionated on a silicic acid column (100 mesh) after redissolving them in hexane and eluting them with a succession of non-polar solvents containing increasing amounts of ether in hexane (Dittmer and Wells, 1967). Where the lipid material was insufficient for quantitative analysis the individual neutral lipid classes were estimated by visualization on TLC. The neutral lipids were collected from the column in the following order: hydrocarbons by hexane; sterol and fatty acid esters by hexane-15% benzene; triglycerides by hexane-5% ether; free sterols by hexane-15% ether; pigments by ether.

Thin layer chromatography

All TLC separation was performed on 20 x 20 cm silica gel F-254 plates (E Merck A.G.) obtained from Brinkmann Instruments Inc. One dimensoonal TLC systems used to separate individual lipid classes are listed on Table 1. All column fractions were checked for purity by TLC. The developing solvent used for the sulfatide was chloroform-methanol-water (65:33:1, v/v/v). Phospholipid classes were separated directly using a two-dimensional system developed by Rouser et al, (1969). The plate was developed in the first direction with chloroform-methanol-ammonium hydroxide 7N, (65:35:5, v/v/v), allowed to dry under nitrogen then developed in the second direction with chloroform-acetone-methanol acetic acid-water, (3:4:1:1:0.5, v/v/v/v/v).

Lipids were visualized on the TLC plates by exposing the developed plate to iodine vapor or by charring the lipids directly by heating the developed plate on a hot plate at 180°C after it had been sprayed with a solution of 25% sodium busulfate w/v containing 3% H_2SO_4 (v/v), (Haines, 1965).

The individual lipid classes were determined by identification with specific color reagents, co-chromotography with authentic standards obtained from Supelco Inc. and in the case of sulfatide a synthetic sulfatide provided by Dr. T. H. Haines and comparison of their R_F

Table 1 Solvent systems and R_f values of standards and lipids extracted from plants and separated on TLC

Lipid	Solvent A	Lipid	Solvent B	Lipid	Solvent C
Pigments and neutral lipids	0.99	Pigments and neutral lipids	0.90	Hydrocarbons	0.90
Sterol glycoside fatty acid esters	0.93	Sterol glycoside fatty acid esters	0.79	Carotenes and sterol fatty acid esters	0.85
Monogalactosyldiglyceride and diphosphatidylglycerol	0.83	Monogalactosyldiglyceride	0.67	Fatty acid methyl esters	0.69
Sterol glycosides	0.70	Sterol glycosides and phytocerebrosides	0.45	Triglycerides	0.66
Phosphatidic acid and phytocerebrosides	0.66	Digalactosyldiglyceride	0.26	Free fatty acids	0.42
Digalactosyldiglyceride and phosphatidylethanolamine	0.55	Sulfolipid	0.11	Free sterols and diglycerides	0.15
Phosphatidylglycerol	0.36	Origin, phospholipids		Monoglycerides, chlorophylls and xanthophylls	0.07
Phosphatidylcholine and sulfolipid	0.30			Origin, polar lipids	
Phosphatidylinositol	0.23				
Phosphatidylglycerine	0.09				
Origin					

Solvent A, chloroform-methanol-water 65:25:4; solvent B, acetone-acetic acid-water 100:2:1; solvent C, hexane-diethyl ether-acetic acid 80:20:1. Phytocerebroside have not been conclusively identified. Phytocerebrosides had chromatographic properties similar to calf brain cerebrosides.

values. Color reagents used to identify specific lipids or lipid groups were as follows: Sterols and sterol esters with the antimony test (Weicker, 1959); glycolipid with anthrone reagent (Spiro, 1966) and Diphenylamine reagent (Wagner et al, 1961); phospholipids with Phospray obtained from Supelco Inc., choline phosphatides with Dragendorff reagent (Wagner et al, 1961) and amino phosphatides with Ninhydrin reagent (Skipski et al, 1962); sulfolipids were identified with Sulfospra obtained from Supelco Inc.

When necessary the appropriate visualized spot or zone was scraped off the plate into an ignition tube or a scintered glass filter for elution with the appropriate solvent.

Quantitative analysis of lipids

Individual lipid classes were recovered from the silicic acid column and their weight was determined gravimetrically after the removal of silicic acid by centrifugation and checking their purity by TLC. Glycolipid was determined after hydrolysis for 90 min. in 3N HCl with anthrone reagent procedure of Vance and Sweeley, (1967). The hydrolysate was extracted with chloroform before addition of anthrone reagent to remove spurious chromogens (Kuske and Rosenberg, 1971). Absorption was determined at 620 nm with a Beckman DB spectrophotometer. Galactose and

MGDG (from Supelco Inc.) were used as standards and run through the same procedure as the samples. Phosphatides were separated by two dimensional TLC and phosphorus content quantitated. Each spot within a uniform area of the thin layer plate was scraped into a digestion tube. Perchloric acid (72%)was added to the tube. The tubes were capped with marbles to prevent loss of perchloric acid and the lipid digested by heating for 20 min at 180°C in a sand bath. After cooling, 0.92 ml of water, 0.4 ml of 1.25% ammonium molybdate, and 0.40 ml of 5% ascorbic acid were added to the tube. Color was developed by heating the mixture for 5 min in a boiling water bath. Adsorbant was sedimented by centrifugation and absorption was read on a Hitachi Perkin-Elmer spectrophotometer at 797 nm. Phosphatidyl choline was used as the organic standard and KH_2PO_4 as the inorganic standard. Chlorophyll a was assayed by the procedure of Bruinsma (1961) and other pigments were followed by spectral analysis.

Enzyme assays

All assays were carried out at 37°C. The rate of reaction was linear with respect to time and enzyme concentration under standard assay conditions.

Succinate dehydrogenase (Succinate: (acceptor) oxidoreductase EC 1.3.99.1) was assayed by the method of King (1967). Acid phosphatase (orthophosphoric monoester phosphohydrolase EC 3.1.3.2) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3) were

routinely assayed at pH 4.8 and 8.6 respectively with p-nitro phenyl phosphate as substrate (Patni et al, 1974).

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) activity was assayed by the colorimetric determination of ortho phosphate (P_I) produced from enzymatic hydrolysis of PP at 37°C. The assay was a modified procedure of Klemme et al, (1971). In addition to the enzyme preparation the reaction mixture contained 70 mM Tris-HCl buffer pH 8.6, 1mM sodium pyrophosphate and 1mM $MgCl_2$. Enzyme activity was terminated by adding 1ml of 10% Trichloroacetic acid and liberation of P_I was estimated colorimetrically.

B-glucosidase (B-D-glucoside glucohydrolase, EC 3.2.1.21. was assayed by a modification of the procedure of Beck and Tappel (Beck and Tappel, 1968) and Aaronson and de Duve (1968). In addition to the enzyme preparation the reaction mixture (1 ml) contained 40 mM citrate-phosphate buffer (pH 5.0) and 5.3mM p-nitro-phenyl B-D-glucoside as substrate. Enzyme activity was terminated by adding 3 ml of glycine buffer (pH 10.7) and liberation of p-nitrophenol estimated colorimetrically at 410nm.

Na^+, K^+ATPase (ATP phosphohydrolase, EC 3.6.1.4.) was assayed by the method of Keetow and Kaneko (1972); B-galactosidase (B-D-galactoside galactohyrolase, 3.2.1.23) by the method of Hughes and Jeanloz (1967); glucose-6-phosphate (D-glucose-6-phosphate phosphohydrolase EC 3.1.3.9.) by the

procedure of Fleischer and Fleischer (1969); 5'-nucleotidase (5' ribonucleotide phosphohydrolase EC 3.1.3.5.) was assayed by the method of Widnell and Unkeless (1960).

Protein was determined by the method of Lowry et al (1951) with bovine serum albumin dissolved in buffer as the standard. For enzyme assay inorganic phosphate was determined colorimetrically by the method of Fisk and Subbarow (1925) or Chen et al (1956).

Microscopy

All cells, cell-free medium and membrane fractions were examined for homogeneity by light and interference microscopy with a Nikon Research microscope. Material to be examined with the electron microscope was fixed in 1% OsO_4 for 1 hour, postfixed with 1% uranyl acetate, dehydrated with alcohol, cleared with propylene oxide and then a 1:1 mixture of propylene oxide and Epon in that order. A 1 mm piece of the material prepared in this manner was deposited into the tip of a labeled beem capsule and centered with a dissecting needle, the capsule was filled to the top with freshly prepared Epon and dried in an oven at 60°C for 48 hours. The material was then sectioned and examined as described by Aaronson et al (1971).

RESULTS

I. Microscopy

A. Subcellular Organelles and Membranes

The cell homogenate (Fraction I), as observed by phase contrast microscopy, contained nuclei, mitochondria, chloroplasts, some small unbroken cells and cell debris. The cell fractions were observed by phase contrast microscopy. Fraction II contained only nuclei and some unbroken cells. Fraction III contained all the whole chloroplasts, some of which were swollen and were in the shape of water wings. When observed under EM each chloroplast appeared as two lobes of lamellar membrane. Fraction IV contained mitochondria with tubular cristea. Cell debris was also found in the fraction. Fractions V and VI contained ribosomes, membrane in the shape of vesicles of various dimensions as observed by electron microscopy but no other organelles. Fraction VII contained no membrane but did contain an orange pigment which formed aggregates of globular spheres when observed with phase contrast microscopy. Fraction VI was further separated into fractions VI A, B, C and D each somewhat different in appearance. Fraction VI A also contained the pigment abundant in Fraction VII; the pigment appeared amorphous under EM. Fraction VI B contained membrane which appeared as if it might have been derived from Golgi apparatus. It was smooth with two unit double membranes; no ribosomes were present. Fraction VI C contained some of the membrane observed in VI B

but it contained mostly ribosome-like material. Fraction VI D (Figure 3) appeared as small and large vesicles and membrane fragments which were derived from broken vesicles; ribisomes were absent from Fraction VI D. Green pigment was visible in all fractions except Fraction VI D which appeared light brown and in Fractions VII and VI A in which the orange pigment was visible.

B. Cell-free medium and extracellular membrane

No whole or broken cells were found in the cell-free medium. The centrifugate of the cell-free medium recovered at 41,000 X g for 60 min contained a mixture of vesicles, microtubules and mastigonemes (Figure 4). The ECM recovered at 105,000 X g appeared as a mass of vesicles with some mastigonemes but no microtubules (Figure 5). The ECM sub-fraction ECM-1 contained vesicles and mastigonemes but no microtubules (Figure 6) while the ECM-2 contained only vesicles (Figure 7).

II. Gross chemistry

A. Whole cell and the subcellular fractions

The chemical nature of the whole cell homogenate (I) and the subcellular fractions (II-VII) is given on Table 2. The relative amount of protein, lipid and carbo-hydrate are given. The % recovery in dry wt of Fraction II-VII/1 was 88.0%; for protein 97.5%; lipid 98.5%; and

Figure 3. Plasma membrane
Fraction VI D X 19,500

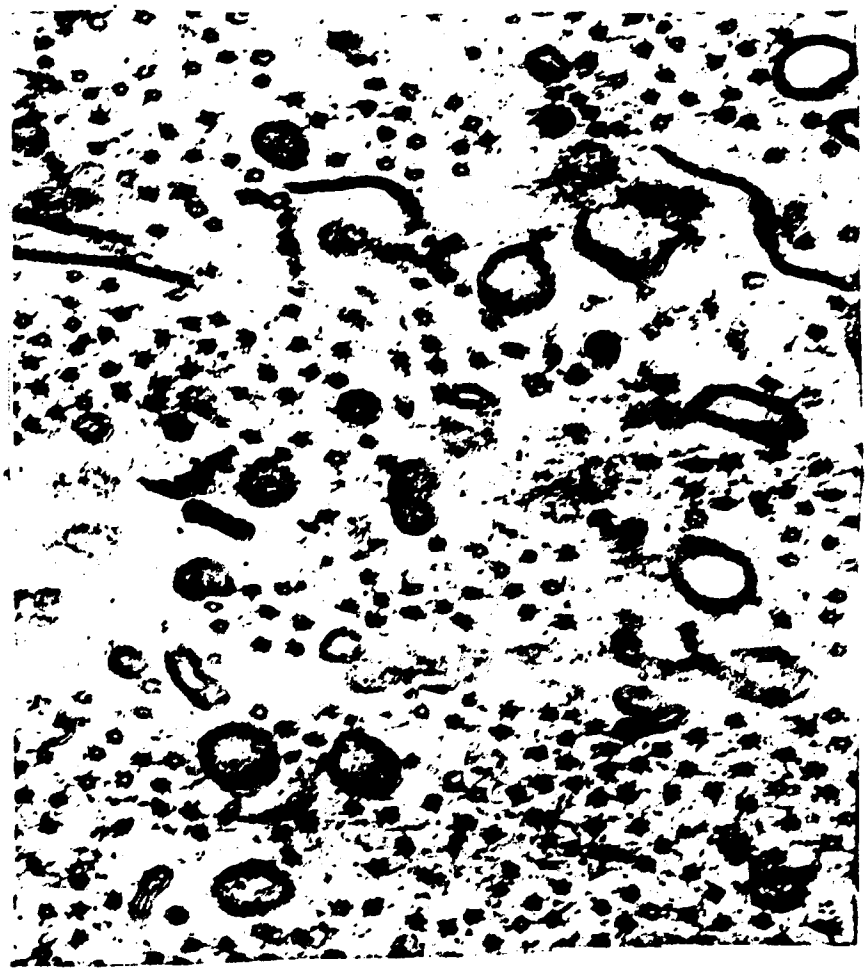


Figure 4. 41,000 X g centrifugate X 39,900

Figure 5. ECM X 39,000

Figure 6. ECM-1 X 19,500

Figure 7. ECM-2 X 19,500

TABLE 2: Gross Chemical analysis of the cell fractions

Dry weight is given as mg and % recovered from (I). Protein lipid and carbohydrate are given as mg and % of their distribution in a specific membrane fraction. The calculated mean and S.D. are based on results of 3-4 experiments.

fraction	Dry weight	Protein	Lipid	Carbohydrate	Dry wt. & % recovery
I mg	2471.1±76	840.0±3.4	610.2±2.0	541.1±1.2	1991.3
%	100	34.0	24.7	21.9	80
II mg	266.1±1.5	86.0±1.1	61.8±2.0	86.0±0.4	233.8
%	12.2	32.3	23.2	32.3	87.8
III mg	385.6±9.0	130.0±0.5	116.0±2.0	102.1±1.6	347.1
%	17.7	33.7	30.1	26.5	90.1
IV mg	262.3±4.0	85.8±0.8	154.3±4.0	4.8±0.9	244.9
%	12.0	32.7	58.8	1.8	93.4
V mg	326.0±20	108.0±2.6	90.8±8.0	36.1±0.9	234.9
%	14.9	33.1	27.8	11.1	72.1
VI mg	431.4±16	128.0±0.7	148.3±6.0	90.2±1.5	366.5
%	19.7	29.7	34.4	20.9	85
VII mg	513.0±10	281.2±6.4	30.3±1.0	202.6±4.3	514.0
%	23.5	54.8	5.9	39.5	100.2

carbohydrate 96.4% (Table 3). The protein content of all fractions was relatively similar except VII in which 54.8% of the dry wt was made up of protein. This fraction also had the lowest lipid content (5.9%) with a lipid/protein ratio of 0.108 (Table 5). Fraction VI had a lipid to protein ratio of 1.13 which is higher than all the membrane fractions except IV. When Fraction VI was subfractionated and the content of VI C and VI D analysed (Table 4), the major component in VI C and VI D was found to be protein (58.9% of the dry wt) while lipids constituted 20.7%. In Fraction VI D lipids made up 47.3% and protein 46.3% of the dry wt. In both fractions there was a decrease in the relative amount of carbohydrate found in VI (20.9%) to 6.4 and 6.8 in VI C and VI D respectively. The material in Fractions VI A and VI B was relatively small, difficult to collect and therefore was not recovered for all analyses. The plasma membrane Fraction VI D, by dry wt represented 68.1% of the unpurified Fraction VI and 13.5% of the original cell dry wt. The % dry wt of the other subcellular fractions of the total cellular dry wt is given in Table 5.

B. Gross chemistry of the ECM, ECM-1 and ECM-2

The gross chemical composition of the ECM and its subfractions was determined (Table 6). The quantity of ECM that was recovered from a two liter culture was 186.6 mgs while 2.4741 grams of cells were recovered. The ECM

TABLE 3: Distribution of the macromolecular components in cell fractions II-VII. Comparison of mg and % recovered of dry weight, protein, lipid and carbohydrate from (I) and their relative distribution among the membrane fractions.

Cell fraction	Dry weight	Protein	Lipid	Carbohydrate
I mg	2471.1	840.0	610.2	541.1
%	100	100	100	100
II mg	266.1	86.0	61.8	86.0
%	12.2	10.5	10.1	15.9
III mg	385.6	130.0	116.0	102.1
%	17.7	15.9	19.0	18.9
IV mg	262.3	85.8	154.3	4.8
%	12.0	10.5	25.3	0.9
V mg	326.0	108.0	90.8	36.1
%	14.9	13.2	14.9	6.7
VI mg	431.4	128.0	148.3	90.2
%	19.7	15.6	24.3	16.6
VII mg	513.0	281.2	30.3	202.6
%	23.5	34.0	5.0	37.5
% recovery	88.0	97.5	98.5	96.4

TABLE 4: Gross chemistry of fractions VIB, VIC and VID. The relative distribution of protein, lipid and carbohydrate in each fraction was given as % of total material recovered. Dry weight represents mg and % of 500 mgs of fraction VI recovered in each fraction after subfractionation.

Fraction		Dry Weight	Protein	Lipid	Carbohydrate	% Recovery
VI	mg	490.0				
	%	99.5	29.7	34.4	20.9	85.0
*VIA		-	-	-	-	-
VIB	mg	19.2±1				
	%	3.9	-	-	-	-
VIC	mg	135.2±5				
	%	27.5	58.9	20.7	6.4	85.0
VID	mg	335.6±18				
	%	68.1	47.3	46.3	6.8	100.4

* Insufficient material recovered for analysis

TABLE 5: Comparison of lipid: protein ratios in cell fractions of O. danica.

Fraction number	% dry wt. of fraction (I)	*Lipid/ Protein
I	100	0.726
II	12.2	0.718
III	17.7	0.893
IV	12.0	1.798
V	14.9	0.609
VI	19.7	1.158
VII	23.5	0.108
VIB	0.8	-
VIC	5.3	0.351
VID	13.4	0.979

* Lipid was determined by dry wt. and Protein determined using BSA as standard

TABLE 6: Gross chemical composition of the ECM, ECM-1 and ECM-2 compared with that of the whole cell homogenate (I). Protein, lipid and carbohydrate are given as mgs and % of their distribution in each fraction. Dry weights are given as mgs recovered from a 2-liter culture and as % and mgs of ECM-1 and ECM-2 recovered from ECM after sucrose density centrifugation. The calculated mean and S.D. are based on the combined results of 4-5 experiments.

Fraction	Dry Weight	Protein	Lipid	Carbohydrate	% Recovery	Lipid/ Protein
I mg	2474.1	840.0	610.2	541.1		0.726
%	-	34.0	24.7	21.9	80.0	
ECM mg	185.6±1.8	76.7±.5	78.3±.5	12.8±.6		1.022
%	100	43.3	42.2	6.9	90.4	
ECM-1 mg	38.4±1.0	13.9±.7	16.1±.8	2.4±0.1		1.152
%	21	36.3	41.8	6.4	84.5	
ECM-2 mg	143.4±1.6	62.7±28	62.1±40	13.0±.7		0.991
%	79	43.7	43.3	8.9	95.9	

recovered therefore constitutes 7.5% of the cell dry wt. After purification on a sucrose gradient the ECM-1 contained 21% or 38.4 mg and the ECM-2 contained 79% or 143.4 mg of the ECM recovered. The % recovery of the ECM was 97.9%. Lipids extracted from these fractions amounted to 78.3 mg or 42.2% for the ECM; 16.1 mg or 41.8% of the ECM-1; and 62.1 mg or 43.3% of the ECM-2. Lipids were found to constitute 24.7% of the whole cell and the ECM was 3.2% of the cell dry wt. The relative amount of lipid in the ECM fraction appeared to be greater than in the whole cell as evidenced by a lipid/protein ratio of 0.726 for the whole cells as compared with 1.022 for the ECM. The quantity of carbohydrates appeared to be 2- to 3-fold greater in the whole cell as compared with the ECM fraction. Since the ECM lipid made up only 3.2% of the cell dry wt, it was necessary to collect the ECM in quantity so that adequate material would be available for microscopic, chemical and enzymatic analyses. A total of fifty 2-liter cultures were used for the collection of the ECM and its subfractions.

III. Lipid chemistry

A. Whole cells

Lipid made up 24.7% of the dry wt of the whole cells. The individual lipid classes were determined (Table 7). The amount of lipid hexose and lipid-phosphorus were determined for each column fraction (Table 8).

TABLE 7: Lipid content of the whole cells. Results are given as mg and % of dry weight of total lipid recovered from a silicic acid column in fractions 1-5.

			SAMPLE NUMBER							
			1		2		3			
Fraction Number	Elution Solvent	Lipid Fraction	mg	%	mg	%	mg	%	Means %	S.D. on %
1	$CHCl_3$	Neutral	57.4	32.6	50.9	29.0	32.6	30.1	30.9	1.84
2	$CHCl_3$-Acetone	"Glycolipid I"	38.0	21.6	41.4	23.6	26.0	24.0	23.1	1.28
3	Acetone	"Glycolipid II"	28.0	15.9	29.2	16.1	15.8	14.6	15.5	0.81
4	CH_3OH	"Phosphatide"	34.5	19.6	36.6	19.3	22.8	21.0	19.9	0.96
5	Butanol	Sulfatide	18.2	10.3	22.7	12.0	11.2	11.3	11.2	0.85
		% Recovery		94.9		98.7		96.6	96.7	1.90

Abbreviations: $CHCl_3$, chloroform; CH_3OH, methanol.

TABLE 8: Phosphorus and hexose content of column fractions of the whole cell lipid (Table 7). The results are given as % of total lipid-phosphorus and lipid-hexose present in each column fraction.

Column Fraction Number	% phosphorus	% hexose
1-NL	1.8	5.2
2-"GL 1"	5.1	54.6
3-"GL II"	10.3	27.8
4-"PL"	80.6	12.4
5-SL	3.2	0.0

When lipid phosphorus was measured for whole cell lipid, 44% of fraction 4 was rich in phospholipids. This led to a revision of the amount of phospholipid in fraction 4 to 8.69% of the total cellular lipid and the sulfatide in the total extract to 21.6%. 80.6% of the total phospholipid was found in fraction 4 and the recovery of that material added to the column was 97.7%.

The relative amount of individual neutral lipid classes were determined (Table 9). The major neutral lipids were found to be triglycerides and diglycerides which constituted 37.9% of the neutral lipid dry wt and sterols which made up 26.8%. There was minor contamination of the neutral lipid fraction with phosphorus and hexose, however chromatography of the eluate from the column used to fractionate the neutral lipid showed that the fractions were free of polar lipids.

The major glycolipids found in the whole cell were MGDG which eluted with chloroform-acetone (1:1 v/v) and DGDG, SQDG and two unknown lipids A and B, which eluted with acetone. Lipids A and B (Figure 8) were first reported to be present in Ochromonas by Nichols and Appleby in 1969. Other minor components of this fraction were chromatographically similar to sterol glycosides.

The principle phospholipids found in O. danica were PE, PC and PG. These and other phospholipids identified were quantitated (Table 10). Phosphorus-containing

TABLE 9: Neutral lipid content of the whole cells. Results are given as mg and % of total dry weight of lipid recovered from a silicic acid column.

	Sample Number							
	1		2		3			
lipid class	mg	%	mg	%	mg	%	Mean %	S.D. on %
HC, St-E FAE, Caratene	5.9	12.3	5.1	12.3	3.7	12.4	12.3	0.1
TG, DG	16.6	35.0	16.0	39.0	11.9	39.7	37.9	2.5
FFA	4.5	9.4	3.9	9.4	3.0	10.0	9.6	0.3
Sterols	15.9	31.0	10.5	25.3	7.2	24.1	26.8	3.7
Pigment	5.9	12.3	5.9	14.0	4.1	13.8	13.4	0.9
% Recovery		94		92		99	95	3.6

Abbreviations: HC, hydrocarbons; St-E, sterol esters; FFA, free fatty acids; TG, triblyderides; DG, diglycerides. Identifications were made by migration position on TLC.

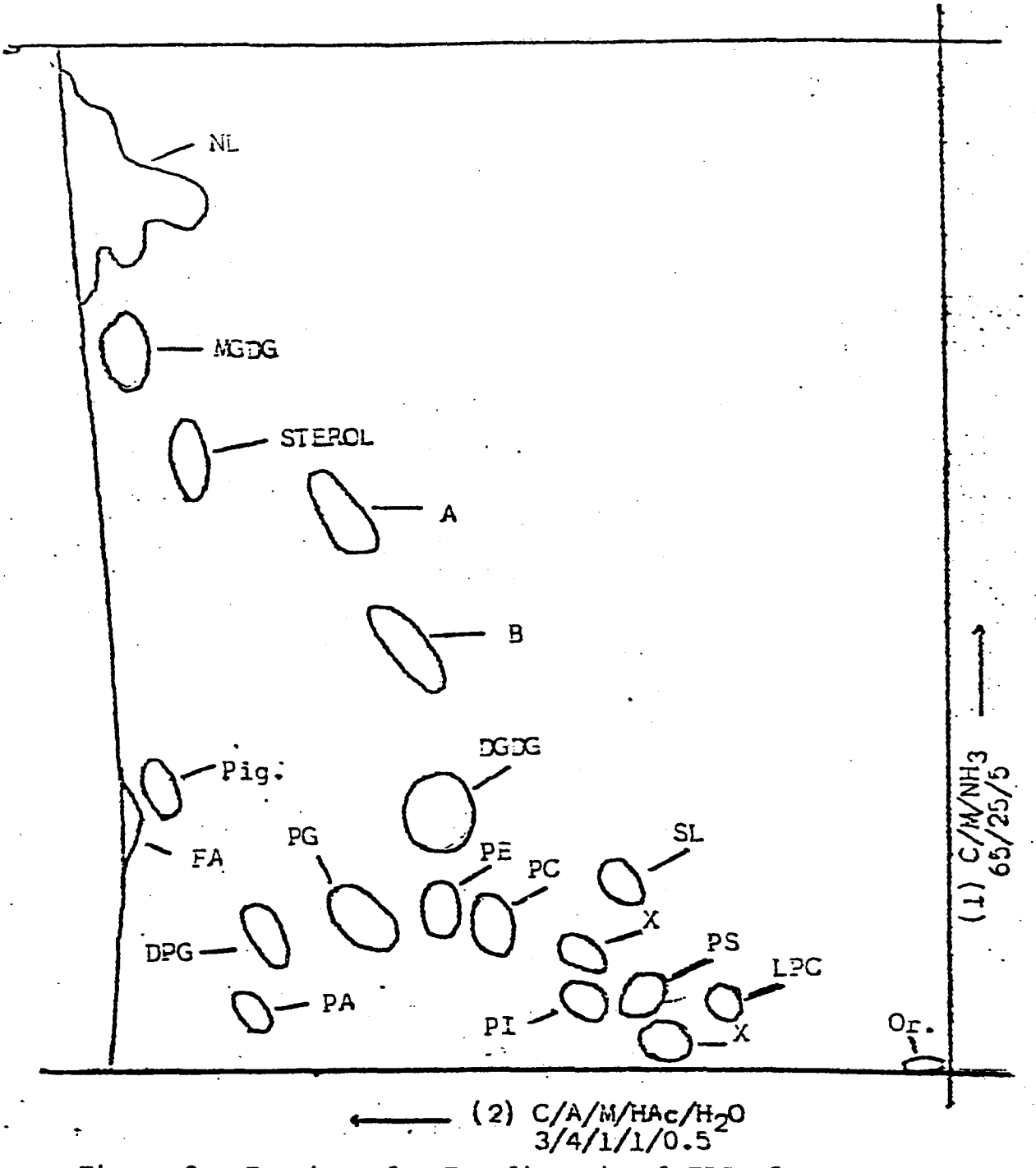


Figure 8. Tracing of a Two-dimensional TLC of some lipids of O. danica. Abbreviations: NL, neutral lipid; FA, fatty acids; MGDG and DGDG, mono- and digalactosyl diglyceride; SL, sulphoquinovosyl diglyceride; DPG and PG, di- and monophosphatidyl glycerol; PA, phosphatidid acid; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PI, phosphatidyl inositol; PS, phosphatidyl serine; LPC, lysophosphatidyl choline; A, B and and X are unidentified compounds.

TABLE 10: Analysis of the phospholipid classes of the whole cell. The phospholipid rich fraction (4) collected off the silicic acid column was further separated into individual phospholipid classes by two-dimensional thin layer chromatography. The separated spots were scraped from the plate and assayed for inorganic phosphorus after digestion with perchloric acid. The results are reported as ug phospholipid-phosphorus recovered.

	SAMPLE NUMBER									
	1		2		3		4			
Phospholipid class	ug	%	ug	%	ug	%	ug	%	Mean %	S.D. on %
LPC	16.6	3.5	14.8	3.2	15.0	3.4	17.0	3.8	3.5	0.3
PC	96.0	20.3	92.0	20.1	104.0	23.8	101.0	22.8	21.8	1.8
PS	40.3	8.5	40.5	8.8	39.5	9.0	41.1	9.3	8.9	0.3
PI	52.0	10.0	50.9	11.1	46.5	10.5	44.8	11.0	10.9	0.3
PE	*225.0	47.5	108.0	23.5	104.0	23.9	109.0	24.7	24.0	0.6
PG	* -	-	105.0	22.9	95.0	21.7	90.0	20.3	21.6	1.3
DPG	-	-	7.6	1.6	7.0	1.5		1.9	1.7	0.2
Other	44.2	9.3	47.5	10.0	26.0	5.9		7.2	8.1	1.9
% Recovery		101.2		98.5		97.2		98.2	98.8	1.7

*On this TLC plate PE & PG did not separate and were collected as one sample.

Abbreviations: LPC, lysophosphatidyl choline; PC, phospatidyl choline; PS, phosphatidyl serine; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; DPG, diphosphatidyl glycerol (cardiolipin)

spots that could not be identified were noted on the TLC plates; these may represent unknown lipids as well as degradation products of the major phospholipid classes. The amount of this material was less than 2% in any one spot and therefore did not contribute significantly to the overall disproportion found between the phospholipids of the whole cell and those of the subcellular or extracellular membrane fractions.

B. Cell-free medium

Lipid material extracted from the cell-free medium was quantitated (Tables 11 and 12). This material represented 7.6% of the cell dry wt of which 69.5% was neutral and 30.5% complex lipid. If sterols are considered membrane constituents then 40.5% of the cell-free medium lipid could be considered principle components of the membranous portion of the cell-free medium. Much of the neutral lipid consisted of free fatty acids and an unidentified neutral lipid. The cell-free medium contained chlorophyll to the extent of 8% of the total lipid. Myelin-like vesicles have been reported to form off the chloroplast of O. danica and were seen in electron micrographs external to and protruding from its cell surface. Such vesicles have also been seen in the 41,000 X g centrifugate (Aaronson et al, 1971) and may account for the chlorophyll found in the cell-free medium.

TABLE 11: Lipids of the cell-free medium of O. danica. The lipid classes are reported as mg dry weight of lipid recovered from the silicic acid column and as % of total lipid recovered in each fraction.

	SAMPLE NUMBER									
	1		2		3		4			
Fraction # & Content	mg	%	mg	%	mg	%	mg	%	Mean %	S.D. on %
1-NL	30.4	66	31.1	67	29.3	66	31.9	71	69.5	2.4
2&3-"GL"	6.7	14	6.5	14	5.7	13	6.2	11	13.0	1.4
4-"PL"	1.7	3.4	1.4	3	1.2	27	1.3	25	2.9	0.4
5-SL	8.3	16.6	7.4	17.6	5.6	13.3	7.7	15.5	15.8	1.8
% Recovery		93		92		99		106	97.5	6.4

Abbreviations: NL, neutral lipid; GL, glycolipid; PL, phospholipid; SL, sulfatide.

TABLE 12: Neutral lipid content of the cell-free medium of O. danica. Neutral lipids were further fractionated on a silicic acid column and are reported as mgs dry weight of lipid recovered and as % of the total lipid recovered.

	SAMPLE NUMBER									
	1		2		3		4			
Lipid	mg	%	mg	%	mg	%	mg	%	Mean %	S.D.
HC, St-E	4.0	13.2	3.7	11.9	2.6	8.9	3.6	11.3	11.3	1.8
Unknown TG	14.8	48.7	13.7	44.1	14.4	49.1	15.1	47.3	47.3	2.3
FFA	4.0	13.2	4.0	12.9	4.4	15.0	4.2	13.3	13.6	1.0
Sterols	4.3	14.1	4.6	14.8	4.8	16.4	4.8	15.0	15.1	1.0
Pigment	3.3	10.8	5.1	16.3	3.5	11.6	4.2	13.2	13.0	2.4

Those lipids thought to be constituents of membrane made up 40.5% of the dry wt of the lipid of the cell-free medium with sulfatide as the major component with 15.8% of the total lipid; glycolipids made up 13%; sterols 10% and; phospholipids 2.9%. The major glycolipids were unknown lipids A and B. The phospholipids were qualitatively similar to those of the cell but were not quantitated due to lack of material.

C. Subcellular membrane and organelle fractions

The nature of the lipid in Fractions II-VII and VI D was examined and was tabulated on Table 13. The membrane free Fraction VII contained only neutral lipid. No complex lipids were recovered from the column in this fraction. The phospholipids constituted 73.1% of Fraction II, 72.5% of Fraction IV, and 55.6% of Fraction V, but only 29.4% and 12.8% of the lipids in Fractions III and VI respectively. While Fractions II-V contained 6% or less, Fraction VI contained 42% of its total lipid as sulfolipid. Glycolipids were highest in Fraction III with 55.5% of the total lipid while Fraction II contained only 4.1%; Fraction IV 8.9%; Fraction V 20.5%; and VI 19.2%. In Fraction VI however the bulk of the glycolipid was in column Fraction 3 and was shown on 2-dimensional TLC to be the unknown lipids A and B and not DGDG or the chloroplast sulfolipid. Unknown lipids A and B appeared on the chromatograms of lipids extracted from Fractions I, V, VI, and VI D but in no other fraction.

TABLE 13: Major lipid classes of the membrane fractions of O. danica. Results are given in % of dry weight of lipid recovered from that placed on column. Mean and S.D. represent the combined results of 3-4 experiments.

	COLUMN FRACTION					
Cell Fraction Number	NL 1	"GL I" 2	"GL II" 3	"PL" 4	SL 5	% RECOVERY
II "Nuclear"	14.4±0.4	4.1±.3	--	73.1±5.6	3.5±.7	91.7±0.8
III "Chloroplast"	13.7±2.5	38.9±25	16.6±2.3	29.4±0.7	1.3±.2	96.7±2.1
IV "Mitochondrial"	15.9±.8	6.7±.6	1.2±.2	72.1±2.6	3.4±.3	97.8±3.8
V "Microsomal I"	17.7±.7	14.7±.3	5.8±.3	55.6±.5	6.1±.9	95.7±2.2
VI Microsomal II"	25.5±.9	2.7±1.2	16.5±1.4	12.8±1	42.4±2.4	94.1±0.7
VII	*100	0.0	0.0	0.0	0.0	
VID Plasma Membrane	22.1±1.9	1.6±.3	19.2±1.6	12.4±1.6	44.5±2.3	100.1±4.3

*Fraction VII found to be free of polar lipid chromatographically

The major neutral lipid in all membrane fractions was sterol with trace amounts of sterol esters, hydrocarbon and pigments (Table 14). The amount of chlorophyll was found to be highest in Fraction III; however this pigment was found throughout the other fractions. A second pigment was found in most fractions (I-VII and VI A). Its absorption spectrum was characteristic of B-carotene and also of extracts of purified eyespot granules isolated from Euglena gracilis (Bartlett et al, 1972). Fraction VII on the other hand, contained a large amount of pigment, triglyceride, free fatty acids as well as sterols, sterol esters and hydrocarbons. The major component of VI D which constituted 44.5% of the total lipid was recovered with the sulfolipid fraction.

The individual phospholipid class profile for each membrane fraction is given on Table 15. There seems to be little variation of PE as % of total phospholipid. There appears to be an enhancement of PC in both VI and VI D over the other membrane fractions while PI and PG are lower in relative abundance in these fractions compared with most of the other membrane fractions. In Fraction III the amount of PG was found to be at least 3 times that of any other membrane fraction. There was no DPG found in this fraction. The % recovery of phospholipid from the TLC plates ranges from 91.7 to 99.2. Other unidentified phosphorus-containing spots on the plates were highest in Fraction II (17.5%) while in other membrane fractions it ranged from 3% in Fraction III to 14% in Fraction VI D.

TABLE 14: Major neutral lipid classes of the cell fractions of O. danica. Results are given as % of total neutral lipid estimated to be present in each spot after TLC.

	NEUTRAL LIPID CLASS					
Cell Fraction Number	HC	ST-E FAE	TG,DG	FFA	Sterol	Pigment
II	20.8	6.9	0.0	2.7	62.5	6.9
III	14.6	5.1	0.0	0.0	43.8	36.4
IV	12.6	12.6	0.0	3.1	69.2	2.5
V	16.9	4.0	0.0	0.0	56.5	16.9
VI	11.0	9.8	0.0	0.0	66.7	11.8
VII	20.0	12.0	48.0	12.0	3.0	5.0
VIII	8.5	8.5	0.0	0.0	81.0	0.0

TABLE 15: Phospholipid classes of the cell fractions of O. danica.

The phospholipid rich fractions collected off the silicic acid column were further separated into individual phospholipid classes by two-dimensional thin layer chromatography. The separated spots were scraped from the plate and assayed for inorganic phosphorus after digestion with perchloric acid. The results are reported as ug phospholipid-phosphorus recovered from the plate and as the ug % of the total phospholipid-phosphorus recovered. The calculated means ± S.D. are based on combined results of 3-4 experiments.

	PHOSPHOLIPID CLASS								
Fraction Number	LPC	PC	PS	PI	PE	PG	DPG	OTHER	% RECOVERY
II	5±1.4	27±1.4	9±0.0	12.5±2.1	25.5±2.1	0.0	3±1.4	17.5±2.1	99.2±4
III	2.7±.6	19.3±2.3	1.3±1.2	8.7±1.2	22±2.0	33±3	0.0	3.0±1.0	95.7±3
IV	2±1.4	25.6±1.5	5.7±1.2	11.3±.6	26.3±.6	2.3±.2	17.7±1	5.3±1.5	93±3
V	3.6±1.2	27.3±2.5	9.3±1.2	10.0±.2	27.7±3.5	8.3±.2	11.3±5	8.3±2.8	97.3±5
*VII VID	5.7±.5	43.7±2.1	6.3±.5	3.6±0.1	21.7±2.1	0.7±.5	4±1.0	14.3±3.2	98.3±5

*No phospholipids were found in fraction VII

D. Extracellular membrane

The ECM and its subfractions ECM-1 and ECM-2 contain predominantly membrane-associated lipids (Table 16). Sulfatide was the major complex lipid in the ECM-2 while the phospholipids were the major group of lipids in the ECM-1. The neutral lipids found in the ECM were sterol esters, hydrocarbons and free sterols along with chlorophyll and an orange pigment. Not even trace amounts of free fatty acids or tri- or diglycerides were found (Table 17). The major neutral lipid in all fractions were the sterols and ranged from 60.2% of the total neutral lipid in the ECM-1 to 81.5% in the ECM. The other neutral lipids combined ranged from 3.7% of the total lipids in the ECM to 7.8% in the ECM-1. Less than 1% of the total lipid was found as chlorophyll in the ECM and all of this segregated with the ECM-1 fraction. The myelin-like vesicles may account for the chlorophyll found in the ECM-1. The absence of these vesicles in the electron micrographs of ECM and ECM-1 suggest that this membrane category is labile and breakdown may occur with successive centrifugation liberating the components into the supernatant. The orange pigment was also found in the ECM and ECM-1.

There was a notable absence of chloroplast glycolipids in the ECM-2 and the main components eluted with the acetone were the two unknowns A and B; this material was also predominant in the glycolipid fraction of the

TABLE 16: Major lipid classes of the ECM, ECM-1 and ECM-2. Results are given as % of total dry weight recovered from the silicic acid column. The calculated means ± S.D. are based on the combined results of 3-4 experiments

	COLUMN FRACTION					
Membrane Fraction	1 Neutral	2 "GL-I"	3 "GL II"	4 PL	5 SL	% RECOVERY
ECM	19.7±1.3	3.0±0.0	15.3±.6	9.1±.2	51.5±1.1	98.7±.7
ECM-1	20.5±2.1	3.5±.7	4.5±.7	30.5±.7	16.0±1.4	75.0±1.4
ECM-2	21.0±1.0	0.0	19.7±1.5	7.0±0	44.7±.6	92.3±2.5

TABLE 17: Neutral lipid classes of the ECM, ECM-1 and ECM-2. Results are given as % of total neutral lipid and (%) of total lipid in the membrane fraction and represent the % estimated to be present in each spot after TLC.

Membrane Fraction	HC	St-E FAE	TG	FFA	STEROLS	PIGMENTS
ECM	6.0 (1.2)	7.6 (1.5)	0.0 (0.0)	0.0 (0.0)	81.5 (16.0)	5.0 (1.0)
ECM-1	15.4 (3.2)	19.6 (4.1)	0.0 (0.0)	0.0 (0.0)	60.2 (12.2)	4.8 (1.0)
ECM-2	15.7 (3.0	9.2 (2.1)	0.0 (0.0)	0.0 (0.0)	72.9 (16.0)	0.0 (0.0)

ECM-1 though there was some MGDG present. The individual phospholipid classes for ECM-1 and ECM-2 were quantitated (Table 18). The relative amount of certain phospholipid classes in the two fractions was found to be different. PC was found to be two-fold higher in the ECM-2 than the ECM-1. PG constituted 11.7% of the total lipid-phosphorus of the ECM-1 and was negligible in the ECM-2. The DPG content in the ECM-2 was double that of the ECM-1. PS, PI and PE content were similar in both fractions. The % of lipid phosphorus-containing spots that were not identified in the ECM-1 was 14.0% while those of the ECM-2 amounted to 3%.

IV. The Plasma Membrane

Electron micrographs of Fraction VI D (Figure 2) revealed the bulk of the fraction to be membrane vesicles and fragments. Subcellular organelle components were seen in all other membrane fractions except VII in which only globular material could be detected.

The plasma membrane was collected above the 45/70% sucrose interphase of a discontinuous sucrose density gradient after centrifugation. This material collected as a dense band surrounded on either side by a brown diffuse border. All this material was collected with the dense band as Fraction VI D. When the VI D fraction was collected and recentrifuged on a gradient with the same specifications the majority of the material was found to collect at the position corresponding to VI D while a minor component collected at a position corresponding to VI C. The density of the

TABLE 18: Phospholipid classes of the ECM-1 and ECM-2. Results are given as % of total phospholipid-phosphorus recovered from the TLC plate. The calculated means ±S.D. represent the combined results of 3 experiments.

MEMBRANE FRACTION	LPC	PC	PS	PI	PE	PG	DPG	OTHER	% RECOVERY
ECM-1	6.3±.6	21.7±1.5	9.7±.6	8.3±.6	24.3±2.1	11.7±6	4.3±6	14±1	95.0±35
ECM-2	9.7±.6	43.0±1.7	6.3±.6	5.7±.6	24.3±2.1	0.3±0	8.3±5	3±.2	99.3±1.6

plasma membrane was estimated to be within the range of from 1.18 to 1.20 g/ml sucrose. Three other bands formed on the gradient which were less dense and microscopically distinct from each other as well as from VI D.

Purity of the plasma membrane of O. danica (Fraction VI D) was estimated by the use of two criteria: enzymic markers, and lipid chemistry.

A. Enzyme composition

Fraction VI contained most but not all of the enzyme markers associated with the plasma membrane: 5'-nucleotidase and Na^+, K^+ - ATPase as well as the largest amount of acid phosphatase, acid B-glucosidase inorganic pyrophosphatase, B-galactosidase and glucose 6-phosphatase (Tables 19 and 20). When VI was purified on a discontinuous sucrose density gradient in which four bands appeared most of the plasma membrane enzyme markers (5'-nucleotidase, Na^+, K^+ - ATPase and B-galactosidase) appeared in Fraction VI D with the acid and alkaline phosphatases (Tables 21 and 22). Further purification of Fraction VI D on a second discontinuous sucrose density gradient gave only two membrane bands at positions comparable to VI C and VI D with most of the plasma membrane activity in the VI D fraction and about 8-28% increase in the specific activity of 5'-nucleotidase and Na^+, K^+ - ATPase. There was no succincic acid dehydrogenase

TABLE: 19

Concentration of molecular markers in the different cell fractions.

Percent represents the distribution of the molecular markers recovered in each fraction based on the total amount initially present in the homogenate (1). The calculated means ± S.D. are based on combined results of 3-6 experiments.

Molecular Marker	Percent recovered in each fraction							Total Recovery
	I	II	III	IV	V	VI	VII	
Protein	100	11.8±1.2	17.1±2.1	10.4±0.7	15.5±2.5	18.8±1.8	14.1±2.1	88
Chlorophyll	100	2.0±0.5	22.0±3.5	3.0±1.5	32.0±4.5	35.0±1.5	4.0±0.5	98
Succinic acid dehydrogenase	100	3.1±0.8	3.1±0.8	76.6±4.5	4.5±1.2	1.5±1.5	0.7±0.5	90
Acid phosphatase	100	1.4±0.3	1.3±0.1	1.5±0.1	9.1±1.6	38.7±0.8	48.2±0.5	100
Alkaline phosphatase	100	3.1±0.3	5.5±0.5	1.9±0.3	7.1±0.6	40.7±4.7	39.6±2.5	98
Acid B-glucosidase	100	2.5±0.9	1.9±0.5	2.4±0.7	18.5±2.1	74.7±5.1	2.5 0.5	102
Inorganic pyrophosphatase	100	8.0±0.5	11.5±2.5	7.5±1.2	20.0±0.8	33.3±1.2	20.5±2.5	101
B-Galactosidase	100	5.1±0.1	1.5±0.1	7.3±0.1	23.5±0.2	62.6±0.4	0.1±0.1	100
Glucose-6-phosphatase	100	2.1±0.1	0.7±0.1	2.2±0.1	1.6±0.2	93.0±0.4	0.4±0.1	100
5' -Nucleotidase	100	6.5±0.5	3.3±3.3	2.9±2.9	8.8±0.5	68.9±5.5	5.5±1.6	96
Na^+, K^+ -ATPase	100	7.5±2.5	7.5±2.5	8.6±0.8	18.4±2.5	42.3±2.8	20.1±1.5	105

TABLE: 20

Specific activity of enzymes in the different cell fractions

Specific activities are expressed as umole of product/min per mg protein. The calculated means ± S.D. are based on combined results of 3-6 experiments.

Enzyme	Fraction*						
	I	II	III	IV	V	VI	VII
Succinic acid dehydrogenase	64.3±10.0	21.9±0.0	14.8±0.5	308.2±10.7 (4.8)	37.1±13.3	5.7±2.4	5.27±0.8
Acid phosphatase	18.1±2.5	3.1±0.5	2.8±0.4	5.7±0.9	29.8±2.4	60.1±0.8 (3.3)	74.7±3.5 (4.1)
Alkaline phosphatase	2.8±0.1	1.2±0.4	1.4±0.3	0.9±0.1	1.8±0.9	3.4±1.0	2.4±0.3
Acid B-Glucosidase	51.1±4.9	18.1±1.5	0.6±1.2	18.4±7.4	69.5±17.4	306.6±9.0 (6.0)	8.1±4.6
Inorganic pyro phosphatase	16.0±2.5	15.6±2.5	14.7±4.5	16.4±1.1	30.9±10.5	58.39±0.4 (3.6)	32.2±1.7
B-Galactosidase	1.98±0.1	0.52±0.1	0.13±0.1	1.5±0.1	1.96±0.2	5.39±0.4 (2.7)	0.05±0.0
Glucose-6-phosphatase	0.70±0.0	0.45±0.1	0.15±0.1	0.49±0.1	0.34±0.2	3.4±0.4 (4.9)	0.09±0.0
5' -Nucleotidase	1.3±0.3	1.7±0.2	0.51±0.1	0.7±0.0	2.1 0.3	6.1±0.1 (3.8)	1.3±0.4
Na^+, K^+ -ATPase	3.5±0.2	1.9±0.5	1.5±0.2	2.0±0.6	6.0±0.6	13.3±1.4 (3.8)	6.7±1.0

Figures in parenthesis represent increase in specific activity over cell homogenate (fraction 1)

TABLE: 21

% Enzyme activity put on sucrose gradient and recovered

Isolated Fraction VI was layered on a sucrose gradient. The gradient was centrifuged at 76,000 xg for 16 hr. The individual bands were removed from the gradient, the membranes isolated and assayed for different enzyme activities. Values as percent recovered represent the distribution of the different enzymes in each fraction based on that initially present in Fraction VI being equal to 100%.

	Top			Bottom	
	Fraction of VI				
Enzyme	A	B	C	D	% Recovery
Succinic acid dehydrogenase	0	0	53.3	2.2	55.5
acid phosphatase	5.8	5.8	8.3	63.8	82.7
Alkaline phosphatase	0	0	0	65.5	65.5
5' -Nucleotidase	0	2.5	40.7	56.3	99.5
Na+ -K+ ATPase	8.8	10.5	20.8	55.4	95.5
B-Galactosidase	0	0	8.00	92.00	100.00
Glucose-6 phosphatase	0	19.8	55.8	24.4	100.00
Acid B-glucosidase	3.9	3.4	69.2	21.8	98.3

TABLE: 22

Specific activity of enzymes in the fractions obtained by discontinuous density gradient centrifugation of the cell membrane (fraction VI)

Specific activities are expressed as umole of product/min per mg. protein. Other details in Table 20.

Enzyme	Top		Bottom	
	Fraction of VI*			
	A	B	C	D
Succinic acid dehydrogenase	0	0	95.2±10.5	3.2±1.5
Acid phosphatase	35.7±.2 (2)	35.7±1.1 (2)	35.5±4.4 (2)	217.7±1.2
Alkaline phosphatase	0	0	0	13.9±1.8 (5)
5' -Nucleotidase	0	0.8±0.8	9.6±1.4 (7)	13.1±0.9
Na^+, K^+ -ATPase	11.6±1.3 (3)	13.1±3.5 (3.7)	27.7±2.2 (8)	52.6±1.6 (16)
B-Galactosidase	0	0	0.71±0.1	7.86±2.5 (3.9)
Glucose-6-phosphatase	0	2.1±0.9	4.4±0.6	1.5±0.2
Acid B-glucosidase	128.4±7.9 (2.5)	108.2±3.1 (2.1)	368.4±10.5 (7.2)	140.5±6.5 (2.8

*Figures in parenthesis represent increase in specific activity over cell homogenate (Fraction 1, Table 20).

activity in the purified Fraction VI D and the specific activity for glucose-6-phosphatase when assayed with 10mM fluoride to inhibit non-specific acid phosphatase was only 0.04%. The plasma membrane preparation obtained after the second discontinuous sucrose density gradient seemed therefore to be free of mitochondrial and endoplasmic reticulum contamination. Most of the succinic acid dehydrogenase (76.6%) appeared in the IV fraction with 4.8 enhancement over the whole cell homogenate. Most of the acid hydrolase activity appeared to be in Fractions V, VI, or VII and most of this activity was not cryptic as only 10% of the activity showed any latency with triton X-100 (Table 23).

B. Lipid chemistry

The lipid profile of the plasma membrane fraction was similar to the other membrane fractions in having as major components complex lipid and sterol. The nature of the complex lipids in Fraction VI D however was different from other subcellular membrane fractions in a number of ways: 1) the relative abundance of sulfatide in the plasma membrane was 5-10 fold higher than in the other membrane fractions; 2) the glycolipid component comprised unknown lipids A and B with a notable absence of chloroplast galactolipids; 3) the phospholipid content was much lower in VI D than was found in the other membrane fractions; 4) the phospholipid profile of VI D was not the same as other membrane fractions. PC

TABLE 23: Latency of acid phosphatase and acid B-glucosidase in different fractions

Enzyme was assayed without (control) and with 0.2% Triton X-100 for latency. The calculated means ± S.D. are based on combined result of 5 experiments.

Cell fraction	Acid Phosphatase		% Latency
	Control	Triton X-100	
I	95.7±9.0	101.1±13.1	5.6
II	7.0±0.7	7.1±0.8	0.4
III	7.4±0.5	7.5±0.3	1.3
IV	8.8±4.1	8.9±4.0	0.4
V	35.1±4.2	38.7±4.9	10.3
VI	173.0±27.0	174.0±27.9	0.5
VII	50.6±5.4	50.6±5.4	0.0
	Acid B-glucosidase*		
I	294.0	300.0	2.0
II	37.6	38.0	1.0
III	31.9	32.1	0.6
IV	35.8	35.8	0.0
V	145.2	151.2	4.1
VI	291.0	308.4	5.9
VII	24.0	24.0	0.0

*mean of 2 experiments

accounted for more than 50% of the identifiable lipid-phosphorus of VI D and there was less than 1% PG in the VI D fraction.

V. Comparison of the plasma membrane (Fraction VI D) with the ECM and its subfractions.

Though pressure homogenization yields a cell homogenate from which intact organelles can be removed by differential centrifugation, there remains many membrane fragments and vesicles which would contaminate the plasma membrane preparation if not removed. Sucrose density gradient centrifugation was therefore used to distribute membrane according to density. The membrane material in Fraction VI was therefore layered on a discontinuous sucrose gradient as described in Methods section. After centrifugation four bands were collected at the following interphases: VI A at 10%/25% sucrose; VI B at 25%/30% sucrose; VI C at 35%/45% sucrose; and VI D at 45%/70% sucrose. The ECM was subfractionated on a gradient with the same specifications used for VI and found to form two bands: ECM-1 at the interphase 25%/30% sucrose and ECM-2 at the interphase 45%/70% sucrose. All membrane material was collected and washed in preparation for analysis. Density in g/cm^3 as it related to % sucrose was evaluated after centrifugation with density beads. Minor alteration of the relative areas as to % sucrose could be detected during the centrifugation period.

The density of the plasma membrane fraction and the ECM-2 were both calculated to be within the range of 1.18 to 1.20 g/ml sucrose at $0^{o}C$ (Figure 9).

Fractionation of the membrane preparations VI and ECM into four and two centrifugally different fractions was reproducible and demonstrated the heterogeneous nature of the material while pointing up certain similarities between Fractions VI D and ECM-2. The chemical composition of these isolated membrane fractions as they compare with the whole cell was given on Table 24. The total lipid and protein are enriched in Fraction VI D, ECM and ECM-2 compared to the whole cell. Though the lipid concentration of the ECM-1 is enhanced over that of the cells the protein content of this fraction is nearly the same as that for the whole cells. In all the membrane fractions there is a decrease in the carbohydrate content over that of the whole cell carbohydrate.

A. Lipid composition

Lipids were found to comprise 24.7% of the dry wt of the intact cells while the plasma membrane Fraction VI D contained 46.3 and ECM-2 42.2% of the respective membrane dry wt. The lipid to protein ratio of the ECM is 1.022, and the ECM-2 is 0.991. These two membrane fractions are more like VI D (0.914) than the whole cell (0.726).

The distribution of neutral lipid classes in the lipids of VI D, ECM, ECM-1 and ECM-2 were compared to the whole cell neutral lipids in Table 25. The most notable

Figure 9. Density determination of the sucrose gradient density beads. Density was determined before and after centrifugation at 76,000 X g for 16 hours by equilibration of density beads with sucrose.

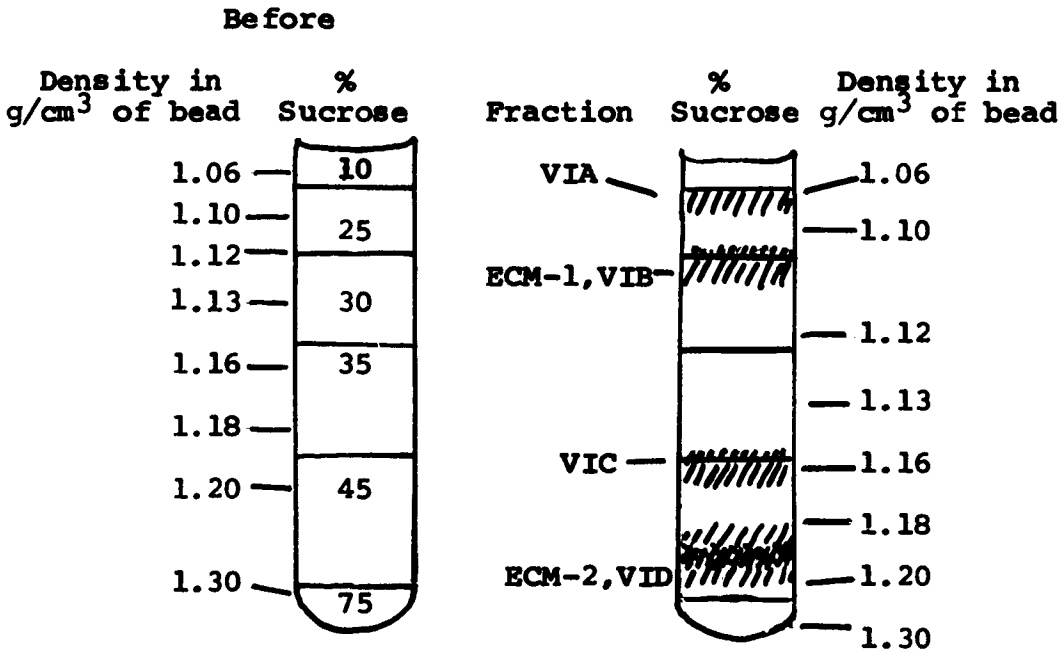


TABLE 24: Comparison of the chemical composition of whole cell plasma membrane fraction (VID), ECM, ECM-1 and ECM-2. Results are given as mg and % of protein, lipid and carbohydrate recovered from the cell and membrane fractions.

FRACTION		DRY WEIGHT	PROTEIN	LIPID	CARBO-HYDRATE	% RE-COVERY	LIPID PROTEIN RATIO
Cells	mg	2474.1	840.0	610.2	541.1		0.726
	%	100	34	24.7	21.9	80.0	
VID	mg	335.6	158.7	155.1	22.8		0.979
	%	100	43.7	46.3	6.8	100.4	
ECM	mg	185.6	76.7	78.3	12.8		1.022
	%	100	43.3	42.2	6.9	90.4	
ECM-1	mg	38.4	139.6	16.1	2.4		1.152
	%	100	36.3	41.8	6.4	84.5	
ECM-2	mg	143.4	62.7	62.1	13.0		0.991
	%	100	43.7	43.3	8.7	95.9	

TABLE 25: Comparison of the neutral lipids of cells with membrane fractions. Results are reported as % dry weight of the total (%TL) and % of the total neutral lipid (%NL). Neutral lipid classes were determined gravimetrically for the cells and were estimated for the membrane fractions.

Membrane Fraction		Neutral Lipid	HC,St-E FAE	TG, DG	FFA	Sterols	Pigments
Cell	(%TL)	31.0	3.8	16.6	3.0	8.3	4.1
	(%NL)	100	11.3	37.9	9.6	26.8	13.4
VID	(%TL)	22.1	4.1	0.0	0.0	18.0	0.0
	(%NL)	100	17.0	0.0	0.0	81.0	0.0
ECM	(%TL)	19.7	2.7	0.0	0.0	16.0	1.0
	(%NL)	100	13.6	0.0	0.0	81.5	4.8
ECM-1	(%TL)	20.5	7.3	0.0	0.0	12.2	1.0
	(%NL)	100	35.0	0.0	0.0	60.2	4.8
ECM-2	(%TL)	21.1	5.1	0.0	0.0	16.0	0.0
	(%NL)	100	25.1	0.0	0.0	72.9	0.0

feature was the large proportion of free sterol in these membrane fractions with lesser amounts of hydrocarbons, sterol esters and fatty acid esters and the complete absence of free fatty acids and triglycerides. The whole cell contained 31% of its lipid as neutral lipid of which free fatty acids and triglycerides constitute 47.5% of the neutral lipids while sterols account for only 26.8%. The neutral lipids of the membrane fractions made up 20% of the total lipid, 10% less than that contributed to the total lipid by the whole cell. Chlorophyll made up 1.0% of the total lipid in the ECM and ECM-1 as compared with 4.1% of the total lipid of the whole cell. No chlorophyll appeared in Fraction VI D or ECM-2. The orange pigment cochromatographed with the hydrocarbons and was found in the ECM-1 and VI A fractions. With respect to the ECM-1 this orange material may not be a component of this fraction but may be a general contaminant collected along with all fractions removed from the top zone of the sucrose gradients.

Complex or more polar lipids comprise 80% of the ECM, 78% of the VI D and 70% of the whole cell total lipids by wt. The relative distribution of glycolipid, phospholipid and sulfatide was quantitated gravimetrically and by analysis of inorganic phosphate and hexose in the above mentioned fractions (Table 26). The purity of these fractions was monitored by TLC. In a small % of the fractions there was overlap. This was reflected in the difference between dry wt. of a particular fraction and the amount of

TABLE 26: Comparison of the major lipid classes recovered from whole cells, plasma membrane, ECM, ECM-1 and ECM-2. The results are given as % of total lipid recovered from the silicic acid column and as % of total lipid phosphorus and hexose in each column fraction.

	Column fraction and lipid classes					
Membrane Fraction	1 NL	2 GL	3 GL	4 PL	5 SL	% Recovery
Whole Cells						
% dry wt.	31.0	23.1	15.5	19.6	11.2	97.7
% P_I	1.8	5.1	10.3	80.6	3.2	
% Hexose	5.2	54.6	27.8	12.4	0.0	
VID						
% dry wt.	22.1	1.6	19.2	12.4	44.5	100.1
% P_I	0.0	0.0	23.3	76.7	0.0	
% Hexose	0.0	0.2	99.0	0.0	0.0	
ECM						
% dry wt.	19.7	3.0	15.3	9.1	51.5	98.7
% P_I	0.0	0.0	0.0	58.8	41.2	
%Hexose	0.0	3.2	96.8	0.0	0.0	
ECM-1						
% dry wt.	20.5	3.5	4.5	30.5	16.0	75.0
% P_I	0.0	0.0	0.0	84.3	15.7	
ECM-2						
% dry wt.	21.0	0.0	19.7	7.0	44.7	92.3
% P_I	0.0	0.0	0.0	100	0.0	

phosphate or hexose material calculated to be present in that fraction.

The majority of the glycolipids were found in column fraction 2, the acetone-chloroform (1:1 v/v) eluate, which contains MGDG and fraction 3, acetone eluate, which contained DGDG, SQDG, and two unidentified lipids A and B. Membrane Fraction VI D was found to contain 1.6 and 19.2% of the total lipid by dry wt. in fractions 2 and 3 respectively. Analysis of hexose in these fractions showed that the content of hexose lipid in fraction 3 and 2 was 99.8% and 0.2% respectively of the total hexose. No hexose was detected in the other column fractions. The ECM lipid contained 96.8% of the hexose in fraction 3 and 3.2% in fraction 2. The dry wt. of fraction 2 and 3, as % of total lipid, in the ECM was 3.0 and 15.3%; ECM-1 was 3.5 and 4.5%; and ECM-2 was 0.0 and 19; these membrane fractions contrast with the whole cell in which 23% of the lipid is found in column fraction 2 and 15.5% in fraction 3. Column fractions 2 and 3 constitute 82.4% of the cells lipid hexose. All five of the glycolipids mentioned above were present in lipid extracts from whole cells, however VI D and ECM-2 did not contain MGDG, DGDG or SQDG, but only lipids A and B. Lipids A and B therefore constitute 19.7% of the total lipid in the ECM-2 and 19.2% of the lipid material in the plasma membrane Fraction VI D.

Phospholipid was found to constitute 10.8% of the lipid of the whole cell as determined by amount of inorganic

phosphate present in the lipids of the whole cell. This was in contrast to the dry wt. of column fraction 4 (19.6%) and suggests an overlap of fractions 4 and 5. Membrane Fraction VI C was found to contain 12.4% and the ECM-2 7% phospholipid by dry wt. The ECM-1 on the other hand contained 30.5% phospholipid. There was also phosphate material found in the acetone eluate of VI D and in column fraction 5 of the ECM and ECM-1. When these amounts were pooled the phospholipid value for VI D was adjusted to 12%, the value for ECM to 14.8% and that for the ECM-1 to 35.1% of the total lipid. The assay for inorganic phosphate in the ECM-2 was 6.9% compared with 7.0% when determined by % dry wt.

Table 27 lists the phospholipids identified from thin layer chromatograms. The effect of phospholipid material material overlapping into adjoining column fractions was taken into account by quantitating all column fractions in which phospholipid was found and not just column fraction four. Considerable variation in the distribution of phosphate among the spots exists between the whole cell, VI D and the ECM subfractions. Notable exception is PE. The % of PC in the ECM-1 (21.7% of the phospholipid) and that of the whole cell (22.0%) are 1/2 that of VI D and ECM-2. Both VI D and ECM-2 have a low PG content. The % of DPG in VI D is closer to the ECM-1 value than that of ECM-2. The % of phosphorus in PS in VI D and ECM-2 is the same but 3 to 5% higher in the ECM-1 and the whole cell phospholipids.

TABLE 27: Comparison of the phospholipid classes of whole cell plasma membrane (VID), ECM-1,and ECM-2. Results are given % of total ug of phospholipid-phosphorus recovered in each spot scraped from the respective TLC plate.

	Phospholipid Class								
Membrane Fraction	LPC	PC	PS	PI	PE	PG	DPG	Other	% Recovery
Cells	3.5	21.8	8.9	10.2	24.0	21.6	1.7	8.1	98.8
VID	5.7	43.7	6.3	3.6	21.7	0.7	4.0	14.3	98.3
ECM-1	6.3	21.7	9.7	8.3	24.3	11.7	4.3	14.0	95.0
ECM-2	9.7	43.0	6.3	5.7	24.3	0.3	8.3	3.0	99.3

The sterol to phospholipid ratio in the whole cells is .769 (Table 28) while that for the plasma membrane Fraction VI D is 1.452 and that for ECM-2 is 2.310. The ECM-1 however, has a sterol/phospholipid ratio of .341 which is closer to that of the organelle fractions than VI D, ECM or ECM-2.

The sulfatides recovered in column fraction 5 constituted 20% of the total cellular lipid, while in the VI D fraction sulfatides accounted for 44.5%. The sulfatide content by dry wt. was also high in the ECM (52.3%) and ECM-2 (48.4%). The content in the ECM-1 was much lower with a value of 16% of the total lipid. There was no cross contamination by glycolipid in fraction 5, however 41.2% of the lipid phosphorus from the ECM lipid was found in column fraction 5 and 15.7% of the lipid phosphorus from ECM-1 was found in that fraction. An adjustment in the amount of sulfatide in the ECM and ECM-1 was made due to the above overlap. In the ECM the sulfatide by dry wt. was adjusted to 45.0% of the total lipid and in the ECM-1 the sulfatide content was adjusted to 10.3% of the total lipid. The sterol to sulfatide ratio in the whole cell, VI D, ECM and ECM-2 were similar and ranged from .355 to .405. This ratio in the ECM-1 was 1.165 and in the organelle fractions range from 2.571 to 4.615 (Table 28).

B. Enzyme composition

The presence of membrane marker enzymes specific

TABLE 28: Lipid ratios of several membrane fractions of O. danica.

	Sterol/ PL	Sterol/ SL	Sterol/ PL+SL
I	0.769	0.384	0.269
II	0.123	2.570	0.117
III	0.204	4.615	0.195
IV	0.152	3.235	0.115
V	0.180	1.638	0.162
VI	1.328	0.401	0.297
VID	1.452	0.404	0.297
ECM	1.081	0.333	0.266
ECM-1	0.341	1.200	0.258
ECM-2	2.310	0.358	0.305

Abbreviations: PL, phospholipid; SL, sulfatide.

for plasma membrane and other cytomembranes was examined with respect to the whole cell, VI D and the ECM. The specific activity in these fractions and the enhancement of activity, if any, is tabulated on Table 29.

5'-nucleotidase was enriched both in VI D and in the ECM 10-12 times over the corresponding specific activity found in the whole cell homogenate. A 3-fold increase in the specific activity of B-galactosidase was found in the ECM while the specific activity found in the VI D fraction was enhances 3.9 times over that of the whole cell.

When Fraction VI D was run for a second time on the sucrose gradient there was no succinic dehydrogenase activity found in the purified Fraction VI D compared to the 176-fold increase in specific activity of succinate dehydrogenase in Fraction IV over the cell homogenate. Absence of this enzyme in VI D was indicative of purification of Fraction VI D with respect to mitochondrial contamination. There was no detectable succinate dehydrogenase activity found in the ECM fraction.

Glucose-6-phosphatase activity in the purified Fraction VI D was only 0.04 when assayed with 10mM fluoride to inhibit non-specific acid phosphatase. There was no detectable glucose-6-phosphatase activity found in the ECM. Both the VI D and ECM did show a 2-fold increase in the specific activity of non-specific phosphatase over that found to be present in the whole cell homogenate.

TABLE 29: Specific activity of enzymes in different cellular and extracellular fractions. The specific activities are expressed as umole of product/min per mg protein. The calculated means ± S.D. are based on the combined results of 3-5 experiments. Figures in parenthesis represent increase in specific activity over cell homogenate (fraction I).

Enzyme	Fraction		
	Cells (I)	Plasma membrane (VID)	Extracellular membrane (ECM)
5' -nucleotidase	1.3 ± 0.3	13.1 ± 0.9 (10)	16.0 ± 0.2 (12)
B-galactosidase	1.98 ± .01	7.86 ± 2.50 (3.9	6.60 ± 0.12 (3.2)
Succinic acid dehydrogenase	64.3 ± 10.0	1.8 ± 2.5	0.0

C. Microscopy

The successive steps in the purification of the extracellular membrane fractions ECM-1 and ECM-2 were monitored by phase and electron microscopy. Whole intact cells were removed at 4,340 X g leaving a supernatant devoid of any cells and with minor cell debris as observed by phase contrast microscopy. There appeared, however to be a degree of flagellar detachment during the initial centrifugation step. This material was recovered along with the above mentioned cellular debris during the second centrifugal step at 41,000 X g. A large number of vesicles and fragments as well as microtubular material has been removed from the supernatant (Figure 4). The ECM recovered at the third centrifugal step, 105,000 x g, is free of microtubular and cellular debris (Figure 5). Figures 7 and 3 compare the membrane vesicles and fragments found in the ECM-2 with those of the plasma membrane Fraction VI D. Both contain vesicles and fragments of similar size and are free from microtubules or cytoplasmic organelles. The bulk of the mastigonemes seem to have been removed during the second step and those that do remain in the ECM appear for the most part to segregate with ECM-1 during the final purification step on the sucrose density gradient.

Discussion

Isolation of the plasma membrane

The technique used to isolate the plasma membrane of O. danica gave a morphologically homogeneous membrane fraction with a density estimated to correspond to 1.18-1.20 g/ml sucrose. The centrifugal behavior of the membrane fragments during the preparative procedure closely paralleled that of Hela cell plasma membrane (Bosmann et al, 1968). The buoyant density of yeast plasma membrane was found to fall within the range of 1.18-1.29 g/ml sucrose (Schibeci et al, 1973) and the plasma membrane of ameba had a density of 1.18 g/ml sucrose (Schultz and Thompson, 1969). Kamat and Wallach (1964) have isolated plasma membrane by first converting cellular membranes into vesicles and then sorting them out by centrifugation. Benedetti and Emmelot (1968) have pointed out however that breakage and refusion of vesicles from different membrane produced hybrid vesicles with hybrid chemical and physical properties. To obviate this problem in my work, cellular membranes were first stabilized with Tris base at pH 7.6 which allowed for the removal from the cell homogenate of intact organelles followed by the assortment of the remaining membrane by isopycnic centrifugation. Other methods used to stabilize membrane employing chemical agents such as FMA, zinc ions or DINB prior to cell disruption (Warren et al, 1966) were avoided because the precise

alteration taking place in the membranes were unknown. Such changes also could alter the chemistry of the membrane and render them unsuitable for enzymatic studies. Membranes isolated with the Tris base method however were suitable for enzymatic studies as no interfering chemicals were present.

Criteria of purity for isolated plasma membranes

In order to evaluate the purity of isolated plasma membranes it was necessary to know what properties distinguished the plasma membrane from intracellular organelles. With respect to photosynthetic cells only the chloroplast and mitochondrial membranes have been investigated (Korn, 1969). The plasma membrane isolated in this work was the first to be isolated from a photosynthetic cell. The isolated plasma membrane from mammalian cells and some eucaryotic microorganisms provided a number of marker criteria (enzymatic and chemical) which were used in evaluating the homogeneity of Ochromonas plasma membrane.

In other instances plasma membrane could be identified because of a special substance, arrangement or structure supporting the membrane; the microtubules were associated with the plasma membrane of the trypanosomatid flagellate Leptomonas collosoma (Hunt and Ellar, 1974) or the pellicle of Paramecium aurelia (Hufnagel, 1969). The plasma membrane of O. danica however lacked a distinguishing

morphological feature by which it could be identified and it was not possible to conclude from the electron micrographs that the plasma membrane was the predominant species in the fraction though homogeneity was demonstrated.

The use of an enzyme marker as a measure of purity required that the enzyme be located solely or mainly in the plasma membrane fraction. Na^+, K^+ - ATPase, 5'-nucleotidase and B-galactosidase were among the several enzymes thought to be useful markers for animal plasma membrane (Korn, 1969). The homogeneity of the O. danica membrane fraction isolated from the sucrose gradient at a density of 1.18-1.20 indicated by electron micrographs is supported by enzymatic analysis (Tables 19-22). The enzymatic markers found in this fraction were similar to the enzymatic markers enhanced in isolated plasma membrane of other cells (Table 30). The increase in specific activity of 5' -nucleotidase, Na^+, K^+ -ATPase and B-galactosidase was similar to that observed in other plasma membranes. In addition to these marker enzymes the plasma membrane contained acid and alkaline phosphatases. Alkaline phosphatase was shown to be a component of the plasma membrane of ameba (Ulsamer et al, 1971). Acid phosphatase was found associated with animal cell lysosomes and has also been reported as a component of the plasma membrane of L. collosoma (Hunt and Ellar, 1974). The specific activity of 5' -nucleotidase of O. danica plasma membrane was very similar to that found in the plasma membrane of ameba and rat liver cells.

TABLE 30: Comparison of marker enzymes found in the plasma membrane of various cells. Values represent enhancement of specific activity in plasma membrane over cell homogenate.

Enzyme	O. danica	L. collosoma (1)	Ameba (2)	Yeast (3)	Hen RBC (4)	Rat Liver (5)	Guinea pig liver (6)
Na^+, K^+ -ATPase	16	4	-	-	10	6	-
5' -nucleotidase	10	2	12	-	2	14-28	4
Acid phosphatase	12	12	-	-	-	-	-
Alkaline phosphatase	5	-	14	-	-	-	5
B-galactosidase	3.9	-	-	-	-	5	-
Mg^{++} -stimulated ATPase	-	-	3	+	-	12	-

(1) Hunt and Ellar, 1974
(2) Ulsamer et al, 1971
(3) Schibeci et al, 1973
(4) Kleinig et al, 1971
(5) Fleischer and Fleischer, 1970
(6) Coleman and Finean, 1966

The absence of detectable amounts of succinic acid dehydrogenase and glucose-6-phosphatase indicated very little contamination from mitochondria or microsomal fractions.

The gross chemical composition of Ochromonas plasma membrane was similar to that of mouse liver, erythrocyte, ameba, and rat liver with respect to lipid/protein ratio and carbohydrate content (Table 31). The phospholipid to protein ratio is much lower in Ochromonas plasma membrane than in the above mentioned membranes. This in part was due to the high sulfatide content of the plasma membrane of O. danica.

One characteristic of the plasma membrane of most eucaryotic organisms is a high ratio of sterols to phospholipid (Coleman and Finean, 1966) compared with the whole cell or other cytomembranes of that cell. The sterol/phospholipid ratio in the plasma membrane of Ochromonas (1.45) was higher than that found in the whole cell or organelle fractions. This value exceeded the highest of a wide range of reported values for the sterol/phospholipid ratio found in the plasma membranes of rat liver cells, L cells and platelets. The sterol/phospholipid ratio of Ochromonas plasma membrane is similar to the values reported for the plasma membrane isolated from intestinal mucosal brush border, Hela cells, lymphocytes and ameba.

TABLE 31: Comparison of the chemical composition of O. danica plasma membrane with that of several mammalian cells.

Membrane	Protein (%)	Lipid (%)	Carbo-hydrate (%)	Ratio of Lipid to Protein	Ratio of Sterol/ PL
O. danica	47.3	46.3	6.8	0.97	1.45
Blood platelet (1)	33-42	58-51	7.5	1.7	0.49
Mouse liver (2)	46	54	2-4	1.15	-
Erythrocyte (3)	49	43	8	0.9	-
Ameba (4)	54	42	4	0.8	0.98
Rat liver (5)	58	42	5-10	0.7	.26-.81
L cells (6)	60	40	5-10	0.67	0.74
Hela cells (7)	60	40	2.4	0.67	1.05
Intestinal brush border cells	-	-	-	0.6	1.26

1 Barber and Jamieson, 1971
2 Evans, 1970
3 Rosenberg and Guidotti, 1968
4 Ulsamer et al, 1971
5 Benedetti and Emmelot, 1968
6 Weinstein et al, 1970
7 Bosmann et al, 1968

The relative distribution of the phospholipid classes in the whole cells of eucaryotic organisms vary (Kates, 1970; Mongnall and Getz, 1973). Ochromonas appears to hold an intermediate position with respect to phospholipid distribution between plant and animal. The PG content of the cells is similar to that of photosynthetic organisms while the DPG content resembles that of animal cells. Such an intermediate position was also suggested by Nichols and Appleby (1969) with respect to the fatty acid distribution within O. danica. The following phospholipids have been encountered in isolated mammalian plasma membranes: phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, cardiolipin, and lyso-derivatives of these. All these compounds were present in Ochromonas plasma membrane as well as trace amounts of phosphatidyl glycerol which may have been due to contamination from chloroplast. The PC and PE content of Ochromonas and rat liver plasma membrane were similar, however, the phospholipids of Ochromonas contained much more PC and much less PE and PS than ameba plasma membrane. The plasma membrane of O. danica and ameba, as well as the whole cell of these organisms, contained no sphingomyelin, a lipid which is enriched in the plasma membrane from mammalian sources.

The absence of appreciable amounts of chloroplast glycolipids from the plasma membrane of O. danica suggest the lack of contamination from this organelle. Galactolipids have been reported as minor components of plant mitochondria

(Schwerter and Biale, 1973) but have not been found in animal mitochondria. Glycolipids such as cerebrosides were found in animal plasma membranes and have been observed in higher plants (Kates, 1970). The exact location of cerebrosides in plant cells is not known. The unidentified lipids A and B were chromatographically similar to cerebrosides and ceramides found in animal cells and reported in lipid extracts of plants (Schwertner and Biale, 1973). Lipid components A and B were major lipid constituents of the plasma membrane of O. danica but were not found to be associated with the organelles. Acetone which eluted lipids A and B also eluted a lipid class that constituted one-third of the lipids found in the plasma membrane of L. collosoma (Hunt and Ellar, 1974) and were chromatographically similar to lipids of Tetrahymena pyriformis (Jonah and Erwin, 1971).

The sulfatides are a unique group of polar lipids that occur only in O. danica and O. malhamensis, though earlier reports indicated sulfatides were widespread in microbes and algae (Haines, 1965). It is possible that this sulfolipid is present in other organisms but that the method of lipid extraction may exclude it as a component. The sulfatides are very polar lipids and partition with the methanol-water phase of the Folch wash. The methanol-water phase is often discarded and with it the sulfatides, if present. The low phospholipid to protein ratio in the plasma membrane of O. danica was due to the high sulfatide

concentration (44.5% of the lipid and 18.9% of the dry weight of the plasma membrane). Sulfatides constitute 20% of the lipid and 4.5% of the dry weight of O. danica. The value of 4.5% was slightly higher than that arrived at by Elovson and Vagelos (1969) who reported that sulfatides made up 3% of the cell dry weight. Sulfatides appear to be concentrated in the plasma membrane as do the unknown lipids A and B and may be considered as molecular markers specific for the plasma membrane of O. danica.

Characterization and source of the extracellular membrane

The production of the extracellular membrane (ECM) by O. danica appeared to be a continuous process that could take place during all stages of the growth phase as inferred from the appearance of vesicles at the cell surface and in the supernatant of light grown cells regardless of the culture age. (Aaronson, 1971). Lysis of cells during the mid/log phase does not appear to occur to any great extent (Meyer, 1974 thesis). Results obtained for the secretion of macromolecules, vitamins and amino acids support this contention (Aaronson, 1971). Meyer has shown that an internal marker enzyme is released at a considerably lower rate than B-glucosidase, a hydrolase known to be secreted from O. danica. Secretion rather than cell lysis is also supported by the relative proportion of lipid classes found in the whole cell as compared with those found in its cell-free medium or the

ECM fractions as well as the gross chemistry of the whole cell and ECM. The proportion of protein to lipid in the whole cells was much lower (0.726) than either the ECM-1 (1.152) or the ECM-2 (0.991). The carbohydrate content in the whole cells was nearly double that of the ECM-2 and more than three times that of the ECM-1. Degradation products of the phospholipids such as phosphatidic acid, free fatty acids or lyso-compounds of the major phospholipids which would be indicative of lipid hydrolysis associated with cell lysis did not increase in appreciable amounts in the cell-free medium when compared with whole cell. The quantity of sulfatide and phosphatidyl choline in those membranes found in the cell-free medium was double that found for the whole cell and these lipids were concentrated in a specific membrane which suggests secretion of membrane rather than general lysis as the source of the membrane.

Morphologically there was no contamination of the cell-free medium with whole or broken cells. The ECM-2 made up 79% of the ECM and was morphologically homogeneous and resembled the plasma membrane.

The enzyme profile of the extracellular membrane was similar to that of the plasma membrane of O. danica. The ECM when fractionated appeared to represent two different membrane types as suggested by their centrifugal patterns and the nature of their lipid components. ECM-1 was less homogeneous in appearance and contained some mastigonemes as

well as vesicles. The sterol to phospholipid ratio of the ECM-1 was more like that of the organelle fractions than the plasma membrane. The sterol to phospholipid ratio of the ECM-2 was like that of the plasma membrane and was due to the high proportion of sulfatide in both membranes. The relative distribution of glycolipid and phospholipid classes were indicative of differences between ECM-1 and ECM-2. ECM-1 contained all the glycolipids found in the whole cell while the ECM-2 contained only unknown lipids A and B which were eluted with acetone.

There appears then to be a similarity between the plasma membrane of Ochromonas danica and the ECM-2 found in the secretions of this organism based on morphological, centrifugal, enzymatic and chemical properties. Contained within both these membranes are specific molecular markers, the sulfatides and as yet to be identified molecules (A and B) eluted with acetone. These marker molecules are not present in any appreciable quantity in other membrane-limited organelle fractions separated from this organism. From these similarities between the two membranes above and the dissimilarities with other O. danica subcellular fractions we may infer that the possible site of genesis of the ECM-2 is the plasma membrane of Ochromonas danica.

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