# THE FLAGELLAR MEMBRANE OF *OCHROMONAS DANICA*:

# ITS CONSTITUTION AND A PROPOSAL FOR ITS STRUCTURE

-by-

ALVIN S. STERN

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

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# A B S T R A C T

## THE FLAGELLAR MEMBRANE OF OCHROMONAS DANICA: ITS CONSTITUTION AND A PROPOSAL FOR ITS STRUCTURE

-by-

ALVIN S. STERN

Adviser: Professor Thomas H. Haines

The isolation and purification of the flagellar membrane by two methods is described. Both procedures were simple, mild, rapid, and produced pure membrane preparations. Flagella suspended in pH 7.5 Tris buffer released its membrane whereas pH 7.5 Tris buffer containing 1 mM EDTA additionally caused the dissociation of the axonemes of the flagella. The isolated membrane preparation contains no phospholipid but had previously been shown to contain instead, a mixture of chlorinated 1, 14-docosanediol - 1, 14 - disulfates as its primary polar lipids.

This requirement for divalent cations for the stability of the membrane was studied. Purified membrane preparations were treated with magnesium ion (4 mM), calcium (4 mM, 10 mM), manganese ion (4 mM), EDTA (0.0045 mM, 0.45 mM), and no metal at all. In each case the preparation showed a normal trilamellar structure

in transmission electron microscopy. With regard to the divalent cations, there was no distinction between 4 mM magnesium, 4 mM manganese,and 10 mM calcium ions. The latter metal ion had a different effect at 4 mM. Half of this preparation was vesicular whereas the remainder appeared as membrane sheets. The 0.0045 mM EDTA preparation showed similar results. An increase to 0.45 mM EDTA yielded an increase in the number of sheets. The absence of both metal ion and EDTA gave almost entirely sheets.

The distribution of anionic sites on the surface of the plasma and flagellar membrane was examined with the use of poly-cationic ferritin (PCF). PCF was located over most of the plasma membrane and the attached flagellar membrane although the particles were lightly scattered. The binding pattern on secreted extra-cellular vesicles revealed randomly distributed clumps of ferritin on their surface. The density of labeling was much higher on these secreted vesicles than on the cell surface. Similar results were observed with cells which had been pre-fixed with 2% glutaraldehyde. Isolated flagellar membrane vesicles showed high intensity binding of PCF than was found on the flagellar membrane. The data suggest that the chlorosulfatides are protected under a polymeric coat on the cell surface.

Freeze-cleavage of the isolated flagellar membrane vesicles demonstrates that the membrane is capable of fracturing down a "cleavage plane." The fracture faces are smooth, relatively free of pits, grooves, and particles.

The divalent cation composition of membrane preparations was studied in order to establish the molar equivalence of calcium

and magnesium to that of sulfate ester in the membrane. Atomic absorption spectroscopy of membrane isolated in 4 mM manganous ion revealed that neither magnesium nor calcium were present in sufficient concentration to bind to the secondary sulfate groups on a one to two basis. When flagellar membrane was isolated in 1 mM EDTA buffer, no metals were detected.

Nitrogen analysis revealed that all of the nitrogen is accounted for by either protein or amino sugar.

Elemental analysis permitted estimation of the composition of an unknown associated with the membrane. It is 61.2% carbon, 5.1% hydrogen and 33.7% oxygen.

Amino acid analysis demonstrated relatively low concentrations of basic amino acids. The analysis suggests that the proteins are acidic.

A model is presented here for the arrangement of the chlorosulfolipids in the flagellar membrane of O. danica.

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Finally, I will thank my wife, Linda, for her support and love throughout my career as a graduate student. It is to her this work is dedicated.

It doesn't matter if you fall down as long as you pick up something from the floor while you get up.

- Dr. O. J. Avery

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# INTRODUCTION

## 1. The Lipid Bilayer Models of Membranes

Biological membranes serve many purposes in living cells. The most obvious is to divide the aqueous space into separate entities. The plasma membrane bounds cells. The inner mitochondrial membrane compartmentalizes both metabolites and enzymes within the cell. It has been recognized for about a century that the plasma membrane acts as a permeability barrier, controlling the exchange of water and solutes between the cell and its environment.

The first clear evidence for a plasma membrane dates from the observations of W. Pfeffer in the 1890's on the differential solubility of cytoplasm (1). Overton's osmotic studies on the root hairs of Hydrocharis mossus ranae were conducted at about the same time, and his experiments showed that permeation of many solutes was proportional to their lipid solubility (2). The emphasis on the role of lipids within biological membranes arose largely from this work. However, at this early stage, no suggestion was presented with regard to the arrangement of the lipid or lipids until the work of Langmuir. Langmuir's studies (3) on monomolecular films at air-water interfaces suggested that the lipid molecules orient themselves in such a way that the polar ends of the molecules associate with the water and the non-polar ends associate with the

air. The importance of these studies is that it was now possible to measure the dimensions of molecules directly. Thus, analytical techniques, coupled with the concepts of Langmuir, enabled deductions to be made regarding the orientation of lipid molecules within a membrane. In 1925, Gorter and Grendel (4) extracted lipids with acetone from a number of what they termed chromocytes--erythrocytes of different mammalian species including dog, sheep, rabbit, guinea pig, goat and man. In all cases, these authors found that the area occupied by the extracted lipids, when spread as a monomolecular film on water and measured by means of a Langmuir trough, was twice the surface area of the corresponding number of erythorcytes prior to lipid extraction. Considering that all the lipid is present in the red cell stroma (synonymous with today's term, ghost), the suggestion of Gorter and Grendel that the lipids in the membrane were arranged as a bimolecular leaflet, with the polar ends of the molecules oriented toward the aqueous environment and the non-polar ends of the lipids facing each other, was entirely reasonable. As shown years later, Gorter and Grendel reached a correct conclusion, but only because their measurements contained compensating errors (5).

Also in 1925, Fricke (6) used the impedance of a preparation of red blood cells to calculate the capacitance of their surfaces. He assumed that the dielectric constant of the membrane was about 3. He calculated the thickness of the membrane to be $3.3 \times 10^{-7}$ cm. This value he translated to about 30 carbon atoms across the hydrophobic region of the membrane if Langmuir's (3) measurements

on fatty acids were correct.

Ten years after Gorter and Grendel made their original suggestion regarding the lipid bilayer nature of the plasma membrane, Danielli and Davson (7) made similar, although in some respects more elaborate, proposals concerning the structure of biological membranes. From surface-tension studies (8) which showed that values obtained at cell surfaces were considerably lower than were considered possible at the time for a lipid surface, Danielli and Davson proposed a model for the cell membrane in which the lipid bilayer is sandwiched between protein. It has since been demonstrated (9) that phospholipids alone can produce low-surface tension values.

Various hypotheses of membrane structure have been proposed which emphasize the transport of hydrophilic substances through membranes (10), the electron microscopic triple-layer pattern with its remarkably constant dimensions (11), and the presence of a large proportion of proteins in mitochondrial inner membrane (12). However, Stoekenius and Engelman (13) have made the point that in no case has the subunit structure for a membrane been established beyond reasonable doubt.

With the increased awareness of the importance of protein in membranes several workers have turned their attention to a consideration of the organization of proteins and the nature of lipid-protein interactions in membranes. For example, models have been postulated by Wallach and Zahler (14) and by Lenard and Singer (15). Using optical rotary dispersion and circular dichroism to investigate

the protein conformations in various membrane preparations, these workers derived models in which a portion of the membrane protein is present in helical conformation as opposed to random coil. Wallach and Zahler (14) suggested that the hydrophilic portions of membrane polypeptides are located on both surfaces of the membrane and are joined by hydrophobic peptide rods which cross the non-polar core of the membrane. Singer and Nicolson (16) have emphasized the concept of a fluid or dynamic nature of membranes. The classical model of Gorter and Grendel described a static membrane which does not allow for the many biological properties of membranes.

In addition, Singer and Nicolson are careful to distinguish between what they term "peripheral" and "integral" proteins of membranes. The former type of proteins are categorized as those which require only mild treatments, such as increase in ionic strength or use of chelating agents, to dissociate the intact molecule from the rest of the membrane. These "peripheral" proteins, examples of which are cytochrome C of mitochondrial membranes and spectrin of erythrocyte membranes, are held to the membrane by weak interactions and, as opposed to the "integral" proteins, are associated primarily with the polar ends of the lipids of the membrane. The "integral" proteins, which constitute the major protein within the membrane, require much more drastic treatments to dissociate them from the membrane than the "peripheral" proteins. The separation of membrane proteins into two classes is believed to be particularly important when considering membrane

structure since the "peripheral" proteins may not be directly relevant to the structural question.

The arrangement of the lipids has been convincingly shown by freeze fracture to be a bilayer, despite suggestions of a micellar occurrence of lipids in cells as well (17). However, it was necessary for a series of elegant experiments to be performed before workers in the field could be persuaded that the cleavage was indeed down the center of the membrane (18). Moor and Muhlethaler (19) had suggested that the outer and inner surfaces of the membrane are revealed by freeze fracture. Branton (20) argued that the cleavage takes place along the interior of the membrane. In order to resolve this dispute, Pinto da Silva and Branton (21) covalently linked ferritin to both sides of the membrane of erythrocyte ghosts and then demonstrated that ferritin was never observed on the fracture faces. Ferritin could, however, be demonstrated on the surface of the membrane following deep etching. In a similar study, in which the human erythrocyte ghost was labeled with fibrous actin, Tillack and and Marchesi (22) confirmed that cleavage takes place within the membrane.

## 2. Artificial Membranes

Two major artificial membrane systems have emerged for the specific exploration of the arrangement of lipids in the membrane

and their function. These are the liposome or unilamellar vesicle and the black film or bilayer membrane (BLM). Although vesicles were frequently the form in which natural membranes have been isolated, it was Kaback and his co-workers (23, 24), who first showed the usefulness of isolated vesicles of natural membranes for transport studies and thereby their viability as reasonable models for structural studies of natural membranes. BLM was developed by Mueller and his co-workers (25). The BLM was formed by placing a droplet of a mixture of phospholipids in a hydrocarbon solvent on a small orifice in a plastic sheet, which separated two compartments filled with an aqueous medium. The solution in the orifice quickly "drained" and a "black membrane" formed.

The BLM and the liposome membranes (26 - 28) have yielded useful information on the probable assembly, stabilization, permeability and molecular motions of the lipid constituents of biological membranes. Since these artificial membranes are of controlled composition, they have permitted productive exploration of membranes with X-rays, NMR, fluorescence, infrared and Raman spectra, ESR, differential scanning calorimetry (DSC), photodichroism and many other physical techniques. The sequencing of several transmembrane proteins and the establishment of the asymmetry of membrane systems have equally emerged as major insights into the fundamental structure of natural membranes.

## 3. Physical Properties of Membranes

Many lines of evidence have indicated that transport and other functions of biological membranes are sharply inhibited as the temperature is lowered below the thermal phase transition from a disordered smectic state to a paracrystalline state (29). This has been explored most effectively by DSC.

This transition is dependent upon the nature of the hydrocarbon chains of the lipids. The introduction of one trans double bond per chain has a negligible effect on the phase transition of a membrane bilayer but the introduction of a cis double bond dramatically decreases the transition temperature of the bilayer.

Recent evidence from deuterium and $^{13}C$ NMR has shown (30, 31) that the first nine to ten carbons (always saturated) of the fatty acid chains are in the trans conformation, and that beyond that point there is a striking increase in the amplitude and the rate of motion for each carbon towards the methyl end of the chain. This applies to each side of the bilayer. This view is entirely consistent with the X-ray data of Engelman (32) which showed that the spacing between the lipid chains is essentially that of a hydrocarbon crystal, namely an hexagonal array. The spacing distance shifts from .417 nm (sharp) to .46 nm (broad) as the temperature of the membrane is raised from below the phase transition into it, but the hexagonal array remains. The arrangement of alkyl chains in an hexagonal array requires that the carbons be in a trans conformation. It may, therefore, be concluded that

the first nine carbons of fatty acids in bilayers (without sterols) are in a paracrystalline state, and that beyond the ninth carbon the chains are in disarray.

The membrane bilayers may now be looked at as two sheets of ionic head groups that are approximately 5.0 nm apart (32) which rest between two highly organized molecular layers. One layer, away from the center of the bilayer, is a highly organized aqueous region; this follows from the water of hydration of all ions in aqueous solution. The other layer, toward the center of the bilayer and including half the distance toward the center, is a rigid region of hydrocarbon in the paracrystalline state. Beyond that rigid block is an interior, disordered, smectic state which extends from about carbon 9 or 10 on one side of the bilayer and spans the interior to the same point on the other side of the bilayer.

This view of membrane bilayers is entirely consistent with the structure, the arrangement, and the role of cholesterol in the bilayer (33). This molecule is rigid in the region of the rings and flexible on its side chain; thus its orientation in the bilayer with the hydroxyl group hydrogen bonded to some portion of the polar head groups would enhance the rigidity of the paracrystalline regions and yet permit flexibility in the interior smectic. It is perhaps not coincidental that the rigid part of the molecule terminates at about carbon 9 of the fatty acid chain.

This view of the membrane that is emerging from the data currently available suggests that the cis double bond at the 9

position, and those more distal as well, are all in the interior smectic or at the border between the two regions. The function of the interior smectic region of membrane bilayers is not known. It is widely assumed to impart liquidity to the membrane.

In the plane of the membrane the lipid molecules, at first thought to be free to move as a solvent, have been shown by Jost (34) and others to be more restricted--especially in the vicinity of membrane protein molecules around which they form an annulus. The restriction on the motion of lipid molecules in the vicinity of a protein may be explained by the sequence of glycophorin as established by Marchesi and his co-workers (35) with respect to the asymmetry of the phospholipids in the erythrocyte membrane. There is a series of 4 arginines and lysines in the protein sequence within the first 9 residues on the inside surface of the membrane. This surface has been shown to contain virtually all of the phosphatidyl serine (the only anionic lipid) in the membrane (36, 37).

## 4. The Flagellar Membrane of Ochromonas danica

Protozoa have proven to be a useful model system for studying membrane structure and biogenesis. Although they strongly resemble the cells of higher animals and plants in their diversity of membrane-rich organelles, they also possess the same advantages found in bacteria with respect to ease of experimental manipulation.

The phytoflagellate, Ochromonas danica, in addition to these advantages contain a substantial quantity of a series of unusual sulfolipids, making then an even more attractive model system to study the structure of membranes. Those compounds (Fig. 1) are 1, 14-docosanediol-1,14-disulfate, and a group of polychloro derivatives with from 1 to 6 chloro groups replacing hydrogen atoms on the chain. These are accompanied by a smaller group of analogous substances, namely, 1, 15-tetracosanediol-1, 15-disulfate which are likewise chlorinated (38-45).

As aliphatic sulfate esters with a second polar group down the chain of the lipid molecule, and as lipids containing halogen atoms replacing hydrogen atoms on the otherwise saturated chain, they are truly unique membrane lipids. There are indications that these compounds are by no means restricted to O. danica. Mercer and Davies (46, 47) have identified these substances in three Chlorophytes (green algae), two Xanthophytes and two Cyanophytes (blue-green algae) in addition to the two Chrysophytes (golden algae) one of which is O. danica. More recently, Liem and Laur (48) have found four new aliphatic sulfates in three Fucacea (brown algae) from the coast of Britany: Pelvetta canaliculata (L), Fucus vesiculosus(L), and Fucus serratios (L). The four aliphatic sulfates are: 1, 18-tricosanediol-1, 18-disulfate; n-tricosanol sulfate; 1, 6-octadecanediol-1, 6-disulfate; and 10-eicosene-1, 8-diol-1,8-disulfate.

The stability of the bilayer is largely based upon the structure of the polar lipids which are present in membranes. All polar lipids consist of hydrocarbon chains which terminate as a methyl

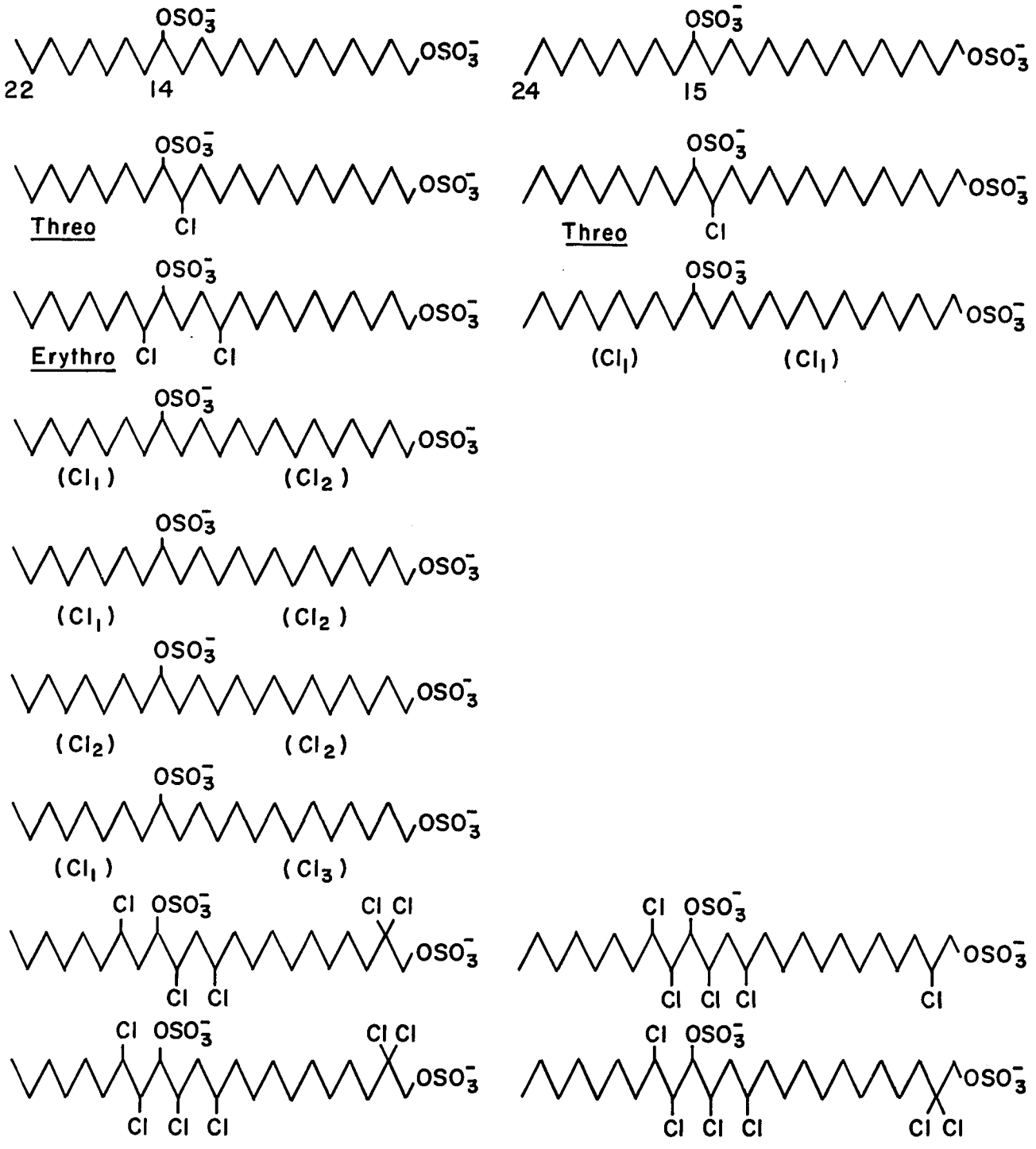



Fig. 1. Structures of the chlorosulfatides described to date

group at one end and a polar hydrophilic group at the other end. The halosulfolipids present an interesting exception to this pattern since they contain a sulfate group at one end of the chain and a second sulfate near the middle of the chain. Additionally, this lipid should not be suitable for forming a monolayer or presumably a bilayer, since it is water soluble.

The flagellar membrane of O. danica has been demonstrated to be uniquely useful for membrane structure studies. Over 90% of the polar lipids of the flagellar membrane consists of the chlorosulfolipids (49). The high content of these substances in a membrane implies that the secondary sulfate is positioned deep in the bilayer of the membrane.

Other features of the flagellar membrane of O. danica makes it amenable to structural studies. It can be easily isolated in high purity by first amputating the flagella from the cells and then separating the sole membrane of the organelle from the axoneme ("9 + 2" structure--Figure 2) by adjusting the pH to 7.5 in the absence of detergents (50). The membrane, which is approximately 8 nm thick in electron micrographs is never found associated with a second membrane, envelope or wall that is visible in electron micrographs. This is typical of flagellar and ciliary membranes and may well be related to a requirement for flexibility. This is of some special significance since the membrane is an unusually thin barrier between the cytoplasm and the outside medium. It shows no osmolality when the fresh water organism is suspended in deionized water. Most cells that do not burst under these

Fig. 2. Diagrammatic representation of flagella ultrastructure

MB: Flagella membrane

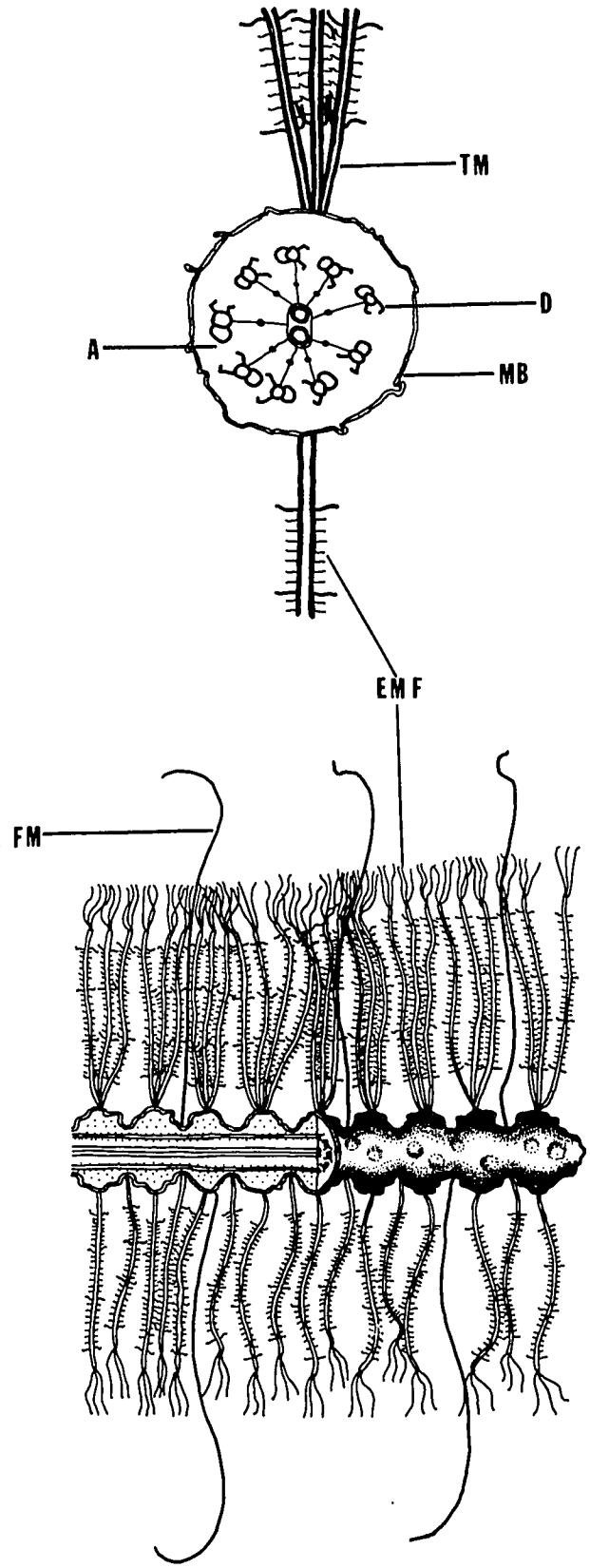
D: Dyneins

A: (9 + 2) Axoneme

TM: Tubular mastigonemes

FM: Fibrous mastigonemes

EMF: Extramastigoneme filaments


TM
D
A
MB
EMF
FM

circumstances are protected by a wall or envelope.

In the case of the fresh water flagellates and ciliates, one might suspect that the lipid composition of the flagellar and ciliary membranes would necessarily be of unusual structures. This is so because such lipids would necessarily be exposed in nature to lipolytic enzymes from other organisms which would likely destroy the membrane. Most microbes are protected by a protein, polysaccharide, glycoprotein or other polymeric coat that would protect them from such hydrolysis. There are only two organisms that lack such protection in which the lipids have been characterized; namely, the cilia of Tetrahymena pyriformis (51 - 58) and the flagella of Ochromonas danica (59), and each have turned out to contain primarily lipids with exotic structure.

As is evident from electron micrographs (60), the flagellar membrane is almost surely continuous with the cell membrane. It is certainly less varied in its proteins and less complicated than the cell membrane. Many of the functions of the cell membrane, e.g.: the transport of most nutrients are likely to be absent.

The surface of the flagellar membrane of O. danica has glycoproteins. As has been shown by Bouck (60) and confirmed by Chen and Haines (50), the mastigonemes (stick-like rods extending from the axonemes (60) with extramastigoneme filaments on them--see Figure 2) consist primarily of glycoprotein. The membrane has been shown to contain at least five major protein bands in SDS-gel electrophoresis (50) all of which are glycoproteins. The sugar composition of both the mastigonemes and the membrane are dominated

by rhamnose (61).

It is likely that some or all of the membrane glycoproteins are recognition sites characteristic of the cell, particularly since the cell membrane and the flagellar membrane appear to be continuous (60). Such recognition sites were studied by Wiese (62) in Chlamydomonas, who found that flagella tips, or isoagglutinins from one mating type can agglutinate cells from the opposite mating type. The mating of Chlamydomonas has been studied extensively (63) and this process is intimately associated with the flagellar membrane and its surface.

In summary, the flagellar membrane of O. danica is the first flagellar membrane that has been explored in any detail. Its lipid composition is unique as may well turn out to be the case with flagellar membranes of all micro-organisms. The uniqueness of the membrane lipids stems from three important structural components:

a. The lipids are alkyl sulfates. As described above, these lipids have only one chain to two sulfate groups. The typical membrane lipid has two hydrophobic chains to one charge.

b. There are from 0 to 6 chloro groups on the aliphatic chain.

c. Perhaps the most unique aspect of these chlorosulfolipids as membrane components is the fact that they contain a secondary sulfate in the middle of the chain. No other

membrane lipid has this structural feature and it must have implications to the architecture of the bilayer of biological membranes.

This study was designed to utilize the unique lipid composition of O. danica's flagellar membrane to obtain a better understanding of the biochemistry and structure of biological membranes. Previously it had been extremely difficult for workers in the membrane field to obtain data about the specific arrangement of structural lipids in biological membranes. The chlorosulfolipids are unusual lipids in their fundamental structural outlines and consequently differ in their physical and chemical properties from the phospholipids, glycolipids and other polar membrane lipids. Nonetheless, they constitute over 90% of the polar lipids of the flagellar membrane of Ochromonas danica (49).

Using this membrane as a model system, the manner in which the lipids are arranged in the membrane was explored.

Is the basic structure of the membrane that of a bilayer? How can secondary sulfates be positioned deep in the bilayer and with what counter-ion? (It is asserted here that the charged sulfate group is deep in the membrane because if both sulfates were at the surface, their bilayer contribution would be less than nine carbon atoms long. Monolayers cannot be made with 8-carbon chains.) How can the polyanionic surface (alkyl sulfate membrane) be stabilized without charge repulsion? How can a biological membrane be made of

a water-soluble detergent?

Three experimental approaches were used to answer these questions:

a. Establish quantitatively the metal cations (sepcifically divalent) in the membrane preparation. This was necessary as the secondary sulfate groups may have been held in the hydrophobic bilayer by ion pairing (to magnesium or calcium) shielding the charges of two secondary sulfates and thereby permitting overall hydrophobicity.

b. Explore the possible presence of an organic nitrogen cation that could shield a charged sulfate in a hydrophobic region. This compound may have been analogous to the lipophosphonoglycan of amoeba (64), or the lipopolysaccharide of Salmonella (65, 66), although these particular polymers have all their nitrogens in amide links. Is there a small divalent organic cation in the membrane preparation? Such an organic cation would necessarily show up in a nitrogen analysis.

c. Study membrane protein composition. In this case an arginine or lysine residue on an "integral" (16) protein might stabilize the secondary sulfate deep in the bilayer.

In order to gain a better understanding of the membrane structure, the membrane was studied under different conditions, i.e., various concentrations of metal cations and EDTA. Electron microscopy was used to observe the character of the membrane vesicles.

Finally, cationized ferritin (67) was used to study the accessibility of the chlorosulfatides to the positively charged protein. These experiments were conducted on (1) whole cells, (2) isolated whole flagella, and (3) the isolated membrane vesicles.

This thesis presents these analytical and micrographic studies on this unique membrane. A model for the arrangement of the chlorosulfolipids in the membrane will be presented.

## EXPERIMENTAL PROCEDURES

### Cultures

Ochromonas danica was grown in the modified chemically defined medium of Aaronson and Baker (68). Unless otherwise stated, the chloride ion concentration of the medium was 0.197 M (Table 1). This concentration was chosen to maximize the amount of hexachlorosulfatides produced while the growth rate was nearly normal.

Inoculations were conducted in ambient light and the cells were cultured at 23.5°C in darkness.

### Flagella Detachment and Isolation (Scheme I)

Cultures of O. danica were harvested 5 days after inoculation by centrifugation at 300 x g for 15 min at 4°C in a Sorvall RC 2-B centrifuge. Cells were washed with fresh media. The cells were then resuspended in fresh media (1/30 of the original culture volume) at 4° and cooled for 1 hour before deflagellation. Flagella were detached by agitation in a 2.5 cm x 10 cm centrifuge tube for 24 sec (3 sec each time, 8 times), at top speed in a Vortex-Genie (Scientific Industries Inc., Springfield, Mass. 01103). Intact and deflagellated cell bodies were then removed from the

Table 1

DEFINED HIGH-CHLORO MEDIA FOR THE GROWTH OF O. DANICA

| | |
|---|---|
| $KH_2PO_4$ | 3.00 g |
| $MgSO_4$-$7H_2O$ | 10.00 g |
| $MgCO_3$ | 4.00 g |
| Ethylenedinitrilo tetraacetic acid | 2.00 g |
| $CaCO_3$ | 0.50 g |
| L-Glutamic acid | 30.00 g |
| Thiamine mononitrate | 0.01 g |
| Dextrose | 100.00 g |
| L-Histidine - HCl | 4.35 g |
| L-Arginine - HCl | 4.03 g |
| Biotin | 0.1 mg |
| Metals mix* | 0.10 g |
| KCl | 2.70 g |
| pH | 4.5 |
| deionized water to | 10 ℓ |

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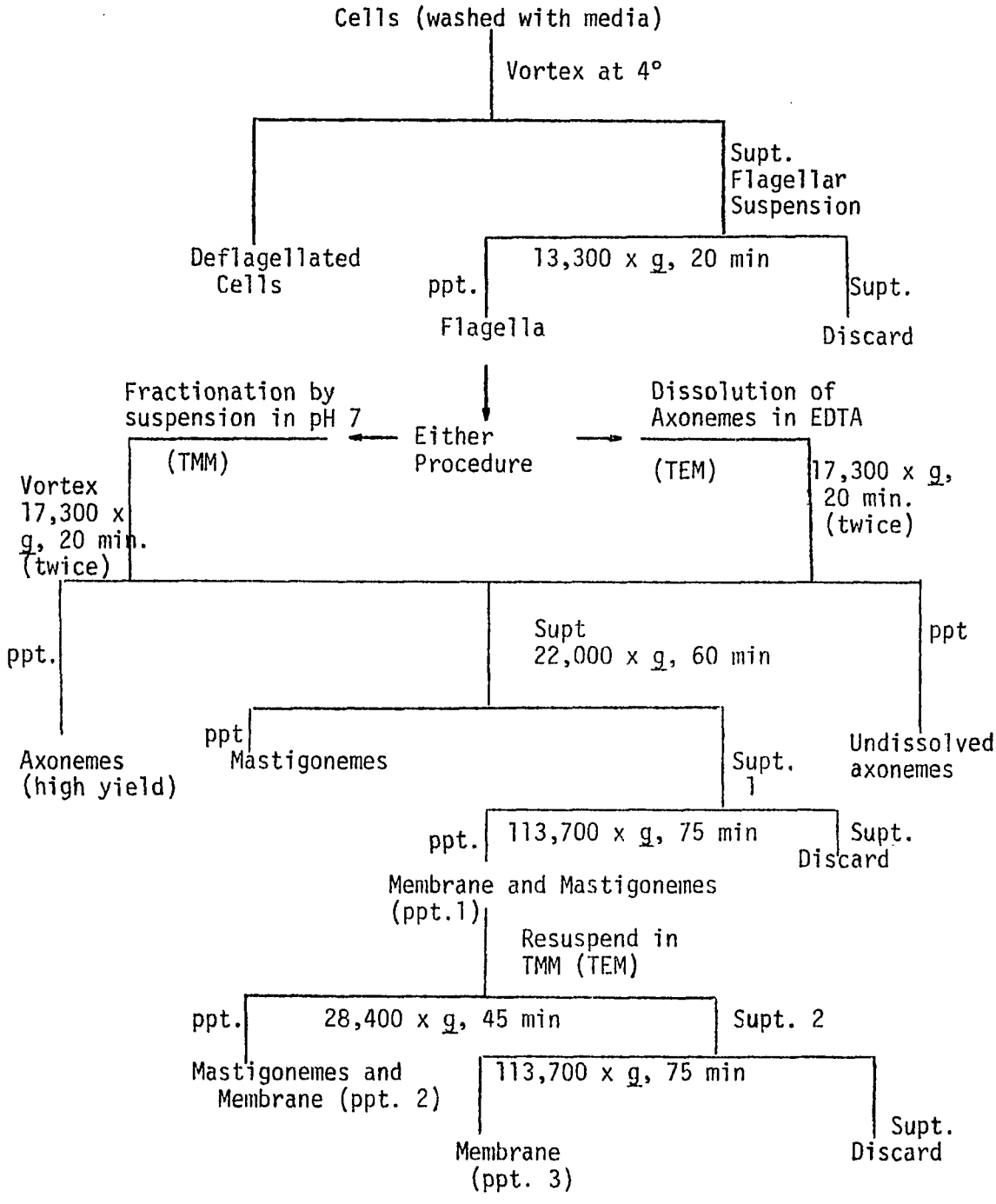
*Metals mix: Contains the following salts: $Fe(NH_4)_2SO_4 \cdot 6H_2O$ 19.9992 g; $ZnSO_4$ . 7 $H_2O$, 9.9991 g; $MnSO_4$ . $H_2O$, 5.0001 g; $CuSO_4$ . 5 $H_2O$, 0.7988 g; $CoSO_4$ . 7 $H_2O$, 1.0010 g; $H_3BO_3$, 1.0002 g; $(NH_4)_6$ $Mo_7O_{24}$ .$4H_2O$, 0.4978 g; $Na_3VO_4$ . 16 $H_2O$, 0.1006 g.

Scheme I

FLAGELLAR MEMBRANE ISOLATION PROCEDURE



medium by centrifugation for 10 min at 208 x g at 4° for a total of three times. The remaining supernatant which contained flagella was centrifuged at 13,300 x g for 20 min in an SS-34 rotor. The pellet obtained was milky white and appeared to be pure flagella under phase contrast and electron microscopy.

Fractionation of Flagella

Flagellar pellets were resuspended in 50 - 100 volumes of TMM[1], stirred overnight and mixed at top speed in a vortex mixer for 30 min to detach the membrane from the axonemes[2] matrix and mastigonemes. This is due to exposure of the flagellar to pH 7 (50).

Dissolution of the Axonemes

This method was used alternatively to obtain a membrane preparation. It took advantage of the stability of the membrane in 1 mM EDTA which dissolved the axonemes.

Flagellar pellets were resuspended in 50 - 100 volumes TEM[1]

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[1]The abbreviations used are: TMM, 10 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mM β-mercaptoethanol; TEM, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (ethylenediamine tetraacetate), 1 mM β-mercaptoethanol.

[2]Axoneme is defined here as a bundle of microtubules, arranged in 9 + 2 pattern, embedded in a matrix.

and stirred for 15 minutes. A suspension of flagella becomes less turbid over the 15-min period as the axoneme goes into the solution.

Flagellar Membrane Isolation

Whether the flagella were fractionated at pH 7.5 (TMM[1]) or suspended in EDTA (TEM[1]) the preparation was then centrifuged at 17,300 x g for 20 minutes at 4°. The supernatant was decanted and recentrifuged at 17,300 x g for 20 minutes at 4°. The pellets were discarded (axonemes) and the supernatant was again centrifuged at 22,000 x g for 1 hour. The resulting pellet contained the bulk of the mastigonemes. A centrifugation force of 113,700 x g was found to convert some membrane sheets and large vesicles into small vesicles (approximately 100 nm in diameter). This conversion apparently allowed the membrane to sediment at a speed different from that of the mastigonemes. Supt. 1 (Scheme I) was therefore centrifuged at 113,700 x g for 75 min in a Beckman Ultracentrifuge Model L 2-65 B (60 Ti rotor). The precipitate (Scheme I, Ppt. 1) was a mixture of mastigonemes and membrane vesicles. This pellet was then resuspended in the appropriate buffer and centrifuged at 28,400 x g for 45 min to precipitate the mastigonemes (Scheme I, Ppt. 2) which also contained some membrane contamination. The supernatant (Scheme I, Supt. 2) was centrifuged at 113,700 x g for 75 minutes. The

precipitate (Scheme I, Ppt. 3) was judged to be pure membrane using electron microscopy as a criterion of flagellar membrane purity (50).

Treatment With Divalent Metals and EDTA

Studies were conducted to determine the stability and electron micrographic properties of the membrane preparation in the presence (and absence) of a variety of divalent cations as well as its tolerance to EDTA. Seven different metal ions and EDTA concentrations were studied. Membrane preparations (Scheme I, Ppt. 1) were resuspended in the following solutions:

TMM; 10 mM Tris-HCl, pH 7.5, containing 4 mM $CaCl_2$, 1 mM β-mercaptoethanol; 10 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$, 1 mM β-mercaptoethanol; 4 mM manganese acetate, pH 6.0; 10 mM Tris-HCl, pH 7.5, containing 0.0045 mM EDTA, 1 mM β-mercaptoethanol; 10 mM Tris-HCl, pH 7.5, containing 0.45 mM EDTA, 1 mM β-mercaptoethanol; 10 mM Tris-HCl, pH 7.5, containing 1 mM β-mercaptoethanol.

Each membrane preparation was left overnight at 4°C. The suspensions were then centrifuged at 113,700 x g for 75 minutes, washed in the respective buffers and fixed for 2 hours with 2% glutaraldehyde (w/v) in 0.2 M cacodylate buffer, pH 7.0, at 4° for electron microscopy.

## Treatment With Polycationic Ferritin (PCF)

PCF (Miles Laboratories, Inc., Elkhart, Ind.) was diluted to 0.50 mg/ml with 0.1 M Na phosphate buffer, pH 7.3. Whole cells, isolated flagella and membrane vesicles were washed in phosphate buffer and then incubated at 25°C for 30 min in PCF. After the incubation, the samples were washed in phosphate buffer and fixed in 2% glutaraldehyde in 0.1 M Na phosphate buffer, pH 7.3

Binding with polycationic ferritin was also carried out on prefixed whole cells. The cells were first fixed in 2% glutaraldehyde in 0.1 M Na phosphate buffer, pH 7.3, for 30 min at 4°. Ammonium chloride was then added to a final concentration of 0.1 M (to block any unreacted aldehyde groups on the cell-bound glutaraldehyde). The cells were washed by centrifuging and resuspending. Binding with PCF was then carried out at 25° for 30 min and the sample was washed with Na phosphate buffer, pH 7.3, for electron microscopy.

## Freeze-Fracture Studies

Membrane vesicles isolated by both the pH 7 fractionation procedure and by the EDTA isolation procedure were fixed for 2 hours in a 1% glutaraldehyde solution and bathed in 20% glycerol for 5 hours. Small pieces of the glycerinated tissue rapidly frozen in liquid Freon 22 and liquid nitrogen were fractured and replicated at -115°C (pressure, $5 \times 10^{-6}$ Torr) on a Balzer's BAF

300 freeze-etch device equipped with a platinum-carbon electron beam gun and quartz thin-film monitor (Balzer's High Vacuum Corp., Santa Ana, Calif.). Platinum-carbon shadowing (2-nm thick film, 45° shadow angle) of the cleaved tissue was performed within 2 sec of the last knife cut and was immediately followed by the deposition of a 20-nm thick carbon coat on the replica. The replicas were cleaned with Clorox bleach, repeatedly rinsed with distilled water, and picked up on Formvas-coated 200-mesh grids. The micrographs are printed as positive images and the shadowing direction is indicated by an arrow in the lower left corner.

Electron Microscopy

All preparations fixed with 2% glutaraldehyde (w/v) in 0.2 M cacodylate buffer, pH 7.0, at 4° or 2% glutaraldehyde (w/v) in 0.1 M Na phosphate buffer, pH 7.3, were post-fixed for 2 hours in 1.0% (w/v) $OsO_4$ prepared in 0.2 M cacodylate buffer, pH 7.0, at 4°. The pellets were sequentially dehydrated with 50, 75, 95, and 100% (v/v) ethanol, cleared in propylene oxide and infiltrated overnight with a mixture of EPON 812 and propylene oxide 1:1 (v/v) and embedded in Epon resin. Silver sections were cut on a Dupont-Sorvall MT-2 ultramicrotome (Du Pont Instruments, Sorvall Operations, Wilmington, DE). The sections were supported on carbon-reinforced collodion covered grids and post-stained with uranyl acetate and lead citrate (69).

All thin sections and freeze-cleave replicas were examined on a Philips 300 electron microscope at 80 kV or on a Hitachi HU-12 electron microscope at 75 kV. Photographs were taken on Kodak Contrast Lantern Slide Plates (Eastern Kodak Co., Rochester, N. Y.).

Negative Staining (Electron Microscopy)

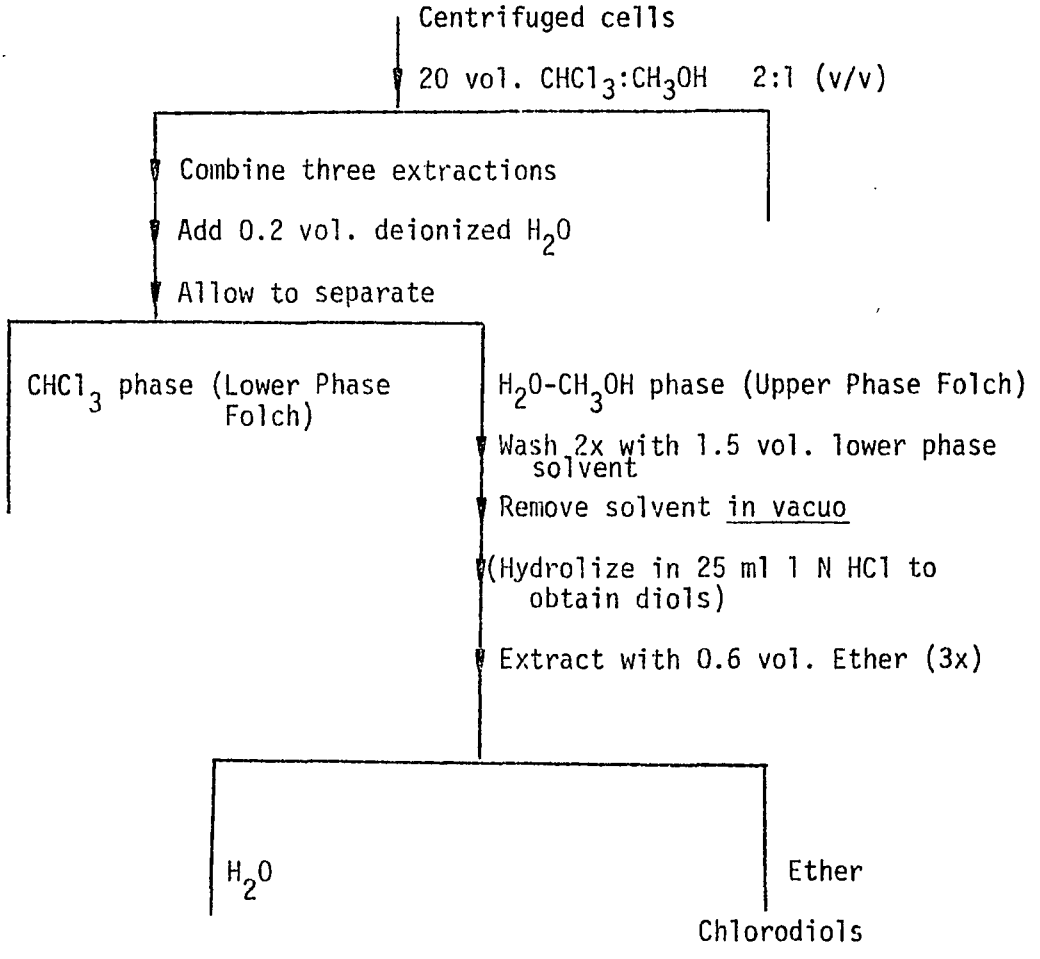
The flagella preparation, resuspended in TEM was diluted with an equal volume of 2% (w/v) solution of ammonium molybdate. Five μl of the mixture was placed on a carbon-reinforced collodion covered grid for 1 min and excess solution was drained off by filter paper. After drying, the grid was examined with a Philips 300 electron microscope.

Isolation of Chlorosulfolipids (Scheme II)

Cells grown in high-chloro (0.197 M) media (Table 1) were extracted with 20 volumes chloroform-methanol (2:1 v/v) by the method of Folch et al. (70) and centrifuged. The extracts were decanted and partitioned against 0.2 volumes of deionized water. The lower phase contained most of the lipid material of the cells. The sulfolipids were in the upper phase of the partioned Folch extract. The contents of the upper phase were taken to dryness in vacuo. The sulfate esters of the crude sulfolipids in the residue were then cleaved by hydrolysis.

Scheme II

ISOLATION OF CHLORO DIOLS



## Preparation of Chlorodiol Mixture

The chlorosulfatides were dissolved in 25 ml of 1 N hydrochloric acid and the mixture was refluxed for 2 hours. The solution was cooled and made basic with 6 N potassium hydroxide. The basic reaction mixture was extracted with an equal volume of diethyl ether. The diethyl ether solution was backwashed with water until neutral, dried over anhydrous magnesium sulfate, and evaporated *in vacuo*. The yield was 3.3 mg of chlorodiols.

## Thin-Layer Chromatography of Chlorodiols

Analytical Silica Gel 60 Plates (Brinkmann Instrucments, Inc.), 20 cm x 20 cm, were activated at 60° for 1 hour, cooled in a dessicator and developed in the first direction with ether: hexane (3:7, v/v). Chromatograms were dried for about 10 min at 60°, cooled in a dessicator and then developed in a second dimension with benzene: chloroform: methanol (50:40:1, v/v/v). The plates were sprayed with 3% (v/v) $H_2SO_4$--25% (w/v) $NaHSO_4$ and charred to visualize the lipids.

## Preparation of Pertrimethylsilyl Derivatives of Diols

Trimethylsilylimidazole in silylation-grade pyridine (Pierce Chemical Company) was added to dried diols and the solution heated in a sealed tube at 67° for 15 min before injection into the gas chromatograph.

## Gas-Liquid Chromatography

Samples were analyzed on an 8 foot stainless steel column of 3% OV - 1 on Chromasorb WHP, 80/100 mesh. The gas-liquid chromatograph was a Perkin Elmer 881 with a flame ionization detector. The helium carrier gas had a flow rate of 60 ml/min. The injector and detector were at least thirty degrees higher than the column.

Trimethylsilyl derivatives of diols were analyzed at 250° for 4 min followed by a linear program of 6°/ min to 320°.

## Elemental Analysis

The elemental analyses were carried out by Schwarzkopf Microanalytical Laboratories (Woodside, N. Y.) using the following procedures:

Carbon, hydrogen: combustion at 900 - 1000° under oxygen followed by gravimetric end analysis; Nitrogen: Kjeldahl with colorimetric end analysis; $SO_4^{2-}$: precipitation as $BaSO_4$; Oxygen: modified Unterzaucher oxygen followed by gravimetric end analysis; Mn, Mg, Ca: atomic absorption spectroscopy; Total metal: combustion at 900 - 1000° followed by gravimetric end analysis.

## Amino Acid Analysis

Hydrolysis of membrane proteins was carried out in heavy walled

tubes which had been washed with a mixture of $H_2SO_4/HNO_3$ (3/1), rinsed in deionized $H_2O$, and oven dried.

Membrane (6.5 mg, containing 1.6 mg protein) was hydrolyzed in vacuo at 110° for 22 hours. The hydrolyzate was dried in a dessicator over NaOH at room temperature to constant weight.

Analyses were performed by Walter Scheppel (Department of Biochemistry, Columbia University) on a Beckman Model 118 Amino Acid Analyzer. All reagents, buffers and resins used in the amino acid analyses were obtained from Beckman Instrument Co.

## The Azure A Colorimetric Assay for Sulfolipid

The procedure of Kean (71) was used. Samples were pipetted into screw cap test tubes and evaporated to less than 0.1 ml volume. To each tube was added 5.0 ml of chloroform: methanol, 1: 1 ( v/v), 5.0 ml of 0.05 N $H_2SO_4$ and 1.0 ml of Azure A solution (40 mg in 5.0 ml of 0.05 N $H_2SO_4$ diluted to 100 ml with water). The tubes were capped, shaken for 30 sec (Vortex) and centrifuged (300 x g for 5 min). The absorbance (Carl Zeiss M4QIII) at 645 nm of the lower phase is a molar measurement using SDS as a standard. It should be noted that the measurements of O. danica sulfolipids (with two sulfates on the molecule) must be halved to obtain molar quantities.

## Protein Assay

The concentration of protein was determined by the method of

Lowry *et al.* (72), using lipid-extracted bovine serum albumin (Sigma Chemical Company) as a standard.

# RESULTS

## Isolation of Flagellar Membrane

Previous isolation of the flagellar membrane of O. danica was achieved by sucrose density gradients which were cumbersome (50). The present procedure utilized only differential centrifugation and the quality and quantity of the membrane preparation was improved. The yield of membrane is 50% by the present method. This was assessed by analysis of the sulfolipid in whole flagella and in the isolated preparations.

A second method for membrane isolation was derived in the present work, although analyses indicated that this method differed from the original method by an absence of inorganic material in the preparation (i.e., no ash on combustion). This new method consisted of dissolving the flagellar axoneme in EDTA (TEM) and separating the membrane from the mastigonemes by differential centrifugation (Scheme I). The yield of membrane is better than 90% with this procedure.

A negatively stained electron micrograph of a suspension of flagella in the EDTA buffer (TEM) is shown in Figure 3. The suspension was revealed to contain tubular and fibrous mastigonemes, membrane vesicles and dissociated microtubules (i.e., tubulin protein)

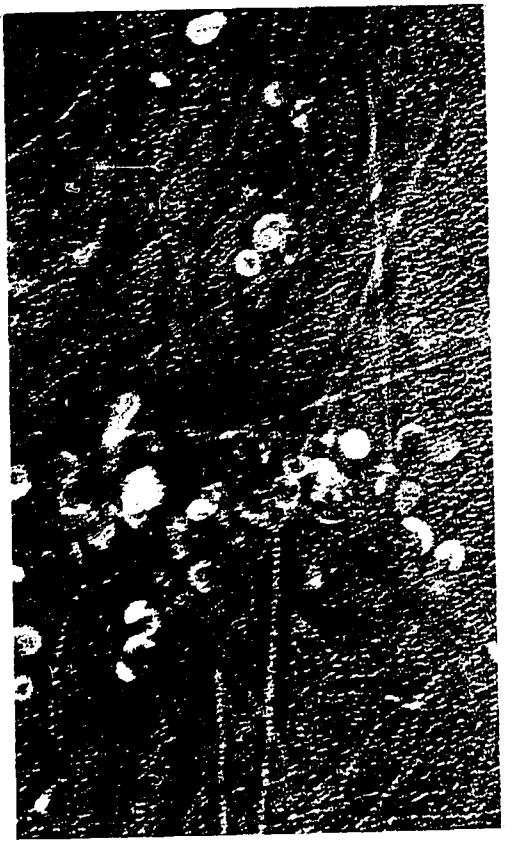


Fig. 3. Electron micrograph of negatively stained (with ammonium molybdate) flagella preparation isolation in buffer containing 1 mM EDTA. After dissociation of axonemes, membranes and mastigonemes are released along with tubulin protein, x 75,000.

of the flagella. The electron micrograph also revealed the presence of occasional undissociated microtubules although the presence of these were quite interspersed. The rods identified as tubular mastigonemes are 20 nm in diameter in agreement with Bouck (60).

Appearance of Flagellar Membrane

The isolated flagellar membrane appeared to be pure, showed a trilamellar structure and consisted mostly of small vesicles (approximately 50 to 150 nm in diameter) as shown in Figure 4. The membrane thickness is about 8 nm.

Stability of the Membrane

The study on the stability of the membrane with regard to divalent cations consisted of a treatment of purified membrane with magnesium ion (4 mM, the concentration normally used for isolation), manganese acetate (4 mM), calcium chloride (4 mM, 10 mM), EDTA (0.0045 mM, 0.45 mM), and no metal ion at all. All of these treatments were at pH 7.5 and in the presence of 10 mM Tris and 1 mM β-mercaptoethanol (except manganese acetate which was at pH 6.0--this being one of the conditions used to isolate the membrane for elemental analysis). Each of the preparations were examined after treatment for 16 hours and numerous electron micrographs were taken. The number of membrane vesicles and the number of membrane sheets were counted and proportioned. Secondly, the length of the membrane

in each conformation was estimated. These two approaches gave identical results with $\pm$ 4% error.

The membrane preparation obtained following the normal procedure is shown in Figure 4. This preparation consisted mostly of small vesicles and showed almost no membrane sheets.

Membrane preparations exposed to 4 mM $Ca^{2+}$ consisted of slightly less than half vesicles (50 to 150 nm in diameter). The remainder of the membrane was short membrane fragments, approximately 150 nm in length (Table 2). Both sheets and vesicles showed a trilamellar structure with a membrane thickness of about 8 nm. The membrane sheets appeared to be popped vesicles as shown by the absence of sheets longer than the circumferance of vesicles. Membrane vesicles exposed to higher concentrations of $Ca^{2+}$ (10 mM) were indistinguishable from the control. The preparation consisted mostly of small vesicles. No membrane sheets were observed in this sample.

After sample exposure to $Mn^{2+}$ (4mM), the vesicular structure of the membrane was maintained (Table 2). These vesicles resembled those isolated in the standard TMM buffer.

The 0.0045 mM EDTA preparation had predominantly vesicles larger than 100 nm. About half of the membrane in the sample was vesicular. The remainder appeared to be popped vesicles which makes the preparation virtually identical to the 4 mM calcium preparation. Both sheets and vesicles show a trilamellar structure with a membrane thickness of about 8 nm. An increase in the EDTA

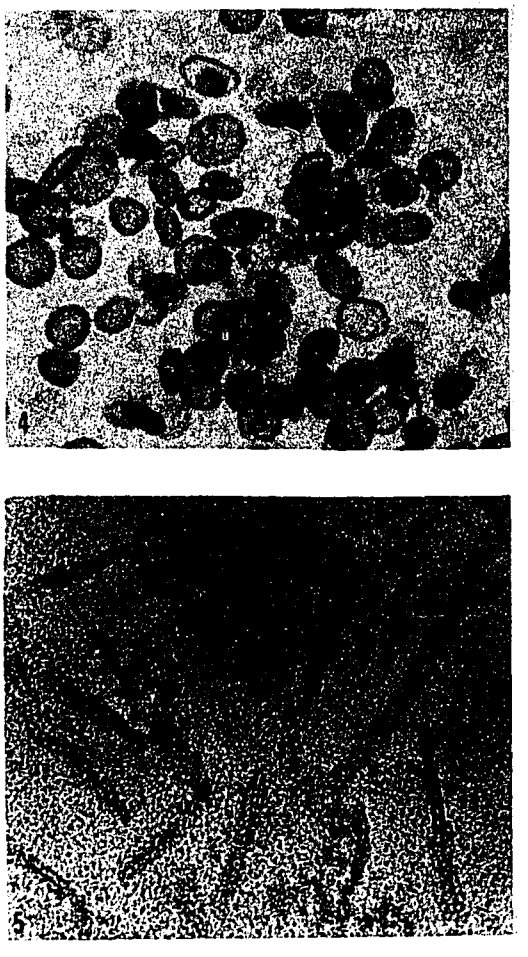


Fig. 4. Electron micrograph of flagellar membrane isolated in TMM after centrifugation at 113,700 x g. x 80,000.

Fig. 5. Electron micrograph of flagellar membrane isolated in buffer containing no metal cations. x 160,000.

Table 2

EFFECT OF METALS ON THE FLAGELLAR MEMBRANE PREPARATION

| TEST SOLUTION | % Membrane Form in Terms of Membrane Number | | % Membrane Form in Terms of Membrane Length | |
|---|---|---|---|---|
| | Vesicles | Sheets | Vesicles | Sheets |
| TMM, pH 7.5 | ~98 | ~2 | ~99 | ~1 |
| 4 mM $CaCl_2$ in 10 mM Tris, 1 mM β-mercaptoethanol, pH 7.5 | 45 | 55 | 44 | 55 |
| 10 mM $CaCl_2$ in 10 mM Tris, 1 mM β-mercaptoethanol, pH 7.5 | ~98 | ~2 | ~99 | ~1 |
| 4 mM Mn-acetate, pH 6.0 | ~95 | ~5 | ~95 | ~5 |
| 0.0045 mM EDTA in 10 mM Tris, 1 mM β-mercaptoethanol, pH 7.5 | 49 | 51 | 52 | 48 |
| 0.45 mM EDTA in 10 mM Tris, 1 mM β-mercaptoethanol, pH 7.5 | 32 | 68 | 32 | 68 |
| 10 mM Tris, 1 mM β-mercaptoethanol, pH 7.5 | 16 | 84 | 20 | 80 |

concentration yielded 68% sheets (Table 2). The preparation consistedof small vesicles (approximately 70 nm in diameter) and both long and short sheets range from approximately 0.08 to 0.88 μm in length.

The elimination of a divalent metal ion in the TMM buffer solution caused the formation of almost entirely membrane sheets (Table 2). The preparation consisted of membrane segments approximately 0.09 μm to 1.0 μm in length (Fig. 5). However, the normal membrane structural characteristics were maintained in this metal ion free buffer.

## Distribution of Anionic Sites on the Plasma and Flagellar Membrane

### I. Anionic Sites on the Outer Cell Surface and on Extracellular Membrane Vesicles

Ochromonas danica has an unusually flexible cell surface capable of producing projections of varying sizes and shapes (Fig. 6). These vesicles have been found in the cell-free growth media (76, 77) and are observed here. Small projections appear to be associated with spherical structures at the surface. Other surface membrane structures were more pointed and did not seem to be associated with spherical surface structures.

When O. danica cells were exposed to PCF (0.50 mg/ml) before glutaraldehyde fixation, PCF was located over most of the cell plasma membranes, although the particles were lightly scattered

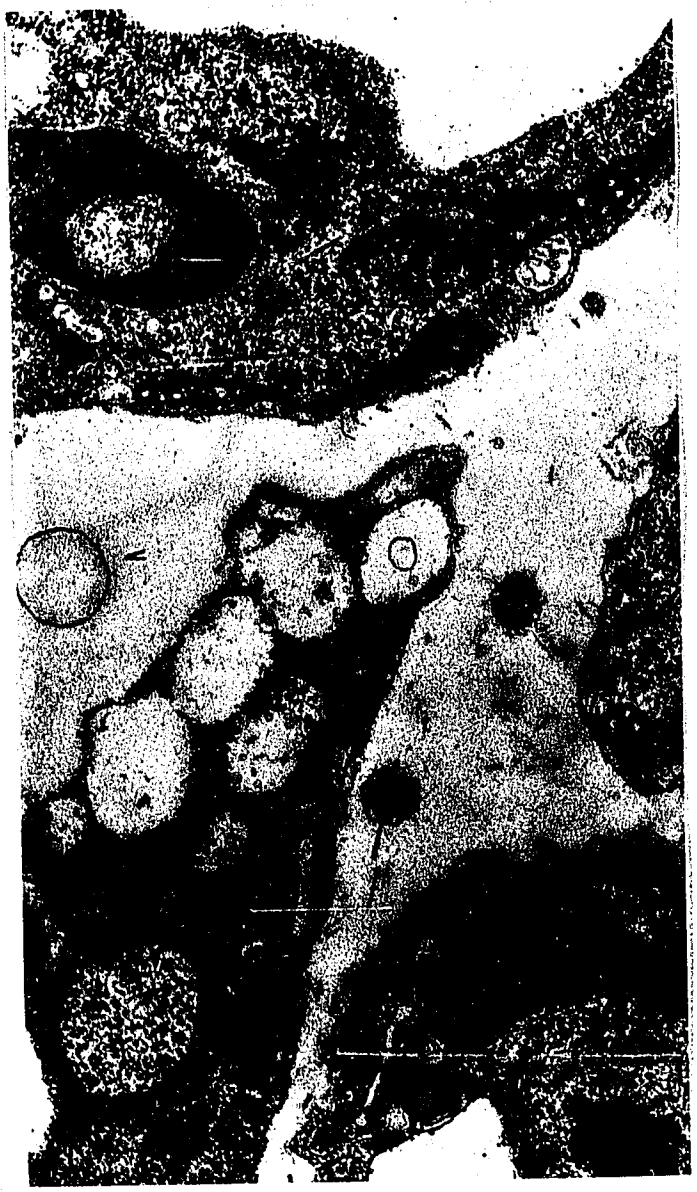


Fig. 6. Ochromonas danica isolated in $NaP_i$ buffer, pH 7.3. The arrow points to membranous structures secreted extracellularly. x 25,000.

(Fig. 7). The binding pattern on the secreted extracellular membrane vesicles was quite distinct (Fig. 7). On these membrane vesicles, PCF was randomly distributed with clumps of ferritin on the surface. The density of labeling was much higher on the secreted vesicles than on the cell surface. PCF covering the cell surface was usually one layer thick and at times seemed to penetrate the bilayer (i.e., the ferritin tended to obscure the ultrastructural details of the membrane).

Similar results were observed with cells which had been pre-fixed with 2% glutaraldehyde (Fig. 8) followed by exposure to PCF (0.50 mg/ml). Whereas a single layer of PCF was bound to some of the cell surfaces, clumps of PCF were observed bound to secreted cell vesicles (Fig. 8). Figure 8 shows the density of labeling was enhanced on the small projections of the cell surface associated with spherical membrane structures exvaginating from the surface.

## II. Anionic Sites on the Flagella

In this experiment flagella were suspended in pH 7.3 buffer. The membrane was therefore detached from the axonemes in some of the flagella. A control for this experiment is shown in Figure 9.

When the flagella were exposed to PCF (0.50 mg/ml), PCF was lightly scattered over most of the attached flagellar membrane (Fig. 10). As usual, on secreted membrane vesicles, the density of PCF binding was much greater. Interestingly, the PCF seems to concentrate at the microtubules, especially the outer nine. This

Fig. 7. A view of Ochromonas danica cell after exposure to 0.50 mg/ml PCF for 30 min. A light coating of PCF is seen on the cell surface. In contrast secreted membranous vesicles (arrow) show PCF binding to surface (see also Fig. 8). x 25,000.

Fig. 8. A view of Ochromonas danica cell surface after fixation followed by exposure to 0.50 mg/ml PCF for 30 min. x 30,000.

Fig. 9. Electron micrograph of isolated flagella. It shows the rippled flagellar membrane. x 78,000.

Fig. 10. Isolated flagella after exposure to 0.50 mg/ml PCF for 30 min. PCF concentrates at the microtubules. x 60,000.

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is consistent with the observations of Danon et al. (67) and Anderson and Hein (79) who observed the effect on flagellar microtubules when PCF was exposed to whole cells.

## III. Anionic Sites of Isolated Flagellar Membrane

Isolated flagellar membrane (Fig. 4) shows a trilamellar structure with a membrane thickness of about 8 nm. When these membrane vesicles were exposed to PCF (0.50 mg/ml), they showed more binding of PCF than was found on the flagella (Fig. 11). The binding pattern consisted of regions of high density similar to that of the extracellular membrane vesicles (Fig. 11). There appeared to be regions of low density binding (as in flagella) and regions of high density binding (as in secreted membrane vesicles). The PCF was also capable of binding vesicles together and clumps of these were observed.

## Freeze-Cleavage Studies on the Flagellar Membrane

It has now been fairly well established that natural membranes exist as a bimolecular leaflet. This has been established for the isolated flagellar membrane of O. danica regardless of the isolation buffer (TMM or TEM) as shown in Fig. 12. The freeze fracture clearly shows the flagellar membrane is capable of fracturing down a "cleavage plane." The fracture faces are relatively smooth,


Fig. 11. Isolated flagellar membrane after exposure to 0.50 mg/ml PCF for 30 min. The binding pattern is similar to that of the extracellular membrane vesicles. x 75,000.

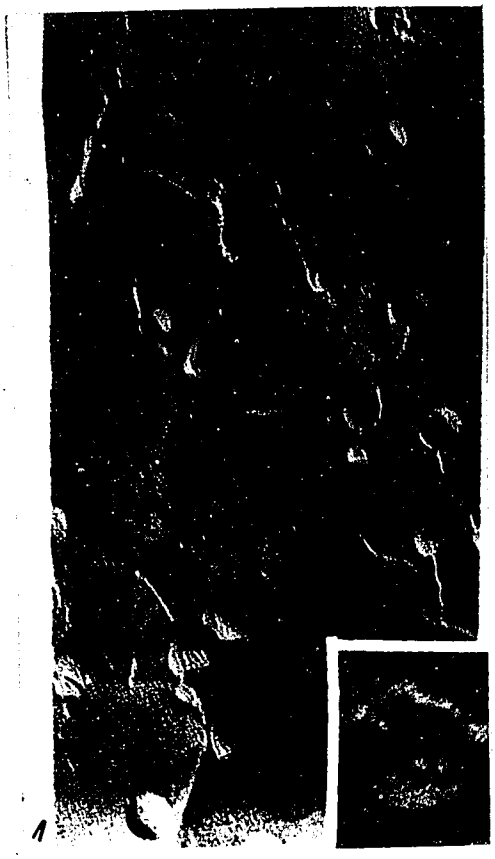


Fig. 12. Freeze cleavage showing the fracture plane of membrane vesicles isolated in TMM. The surfaces are relatively smooth with few pits, grooves, and particles x 74,100. Insert: Two particles in the fracture plane of a vesicle. All the observed particles were approximately 10 nm in diameter as shown. x 150,000.

free of pits, grooves and particles. However, occasionally small clusters of particles of uniform diameter appear on the cleavage plane (Fig. 12, Inset).

## Distribution of Chlorosulfatides

The distribution of the chlorsulfatides in *O. danica* shifts dramatically with the concentration of chloride ion in the growth media. In order to quantitate the shift that occurs in high-chloride media, the chlorosulfolipid was isolated from cells and hydrolyzed. Hydrolysis of the crude sulfatide preparation produces a mixture of diols which are easier to separate and quantitate. Qualitative analysis of the diols on two-dimensional thin-layer chromatography gave results similar to the pattern obtained by Mooney *et al.* (85). The pattern for the hexachlorodiols was particularly pronounced after charring, indicating a significant shift towards the hexachloro compound in the high chloride medium. Mooney *et al.* analyzed cells grown in the normal chloro media (68).

In order to quantitate this shift, pertrimethylsilyl derivatives of the diols were analyzed by gas-liquid chromatography and the molar ratio computed (Table 3). From this ratio, the average number of chloro atoms in the isolated chlorosulfolipids from cells grown in high-chloride media, was calculated to be 2.5. The average molecular weight of the chlorosulfolipids was computed to be 595.8.

Table 3

MOLAR DISTRIBUTION OF DIOLS[a]

| Diol | Molar Ratio |
|---|---|
| Aliphatic diol | 6.4 |
| Monochloro diol | 6.3 |
| Trichloro diol | 2.3 |
| Tetrachloro diol | 1.0 |
| Pentachloro diol | 2.3 |
| Hexachloro diol | 4.6 |

[a]The number of moles of tetrachloro diol is arbitrarily assigned a value of 1.0. Cells were grown in "high-chloride media" (Table 1).

## Elemental Analysis of Flagellar Membrane

Elemental analyses were conducted on three different membrane preparations. Since the three preparations yielded essentially the same results only the analysis on the third sample is shown in Table 4. This sample (unlike the other two) was prepared in EDTA (TEM) buffer--hence the absence of metal ions in the membrane preparation. The first and second preparations were each prepared in 4 mM manganous ion. Analyses were conducted as follows:

> Metal ions were determined by atomic absorption; protein was estimated by total nitrogen (Kjeldahl) less the known amino sugar nitrogen (61) multiplied by 6.38 (this percent weight was confirmed by the method of Lowry et al., (72) using lipid extracted bovine serum albumin as a standard (61)); sulfolipid--based on chloride analysis and calculated from glc analysis of the chlorodiols (this method was confirmed by direct sulfolipid analysis (49) and by direct analysis of ester sulfate using precipitation as $BaSO_4$). The quantitation of sterols, fatty acids and unknown (uncharacterized) lipids (49) is based on the analysis of lipids in the membrane by Chen et al. (49). The specific activities of the three major components of the lipid extract had been determined directly. The specific activities of the fatty acids, sterols, and chlorosulfolipids had been based on average molecular weights of 276, 407

Table 4

ELEMENTAL ANALYSIS

| | Total % Weight | μ Moles in 1 mg. Membr. | C | H | O | N | $SO_4^-$ | $Cl^-$ |
|---|---|---|---|---|---|---|---|---|
| Protein[1] | 21.43 | 0.004 | 9.93 | 1.57 | 6.59 | 3.34 | | |
| Lipids | | | | | | | | |
| I. Sulfolipids[2] | 5.64 | 0.095 | 2.50 | 0.37 | 1.25 | | 1.82 | 0.84 |
| II. Sterols[3] | 0.60 | 0.014 | 0.51 | 0.07 | 0.02 | | | |
| III. Fatty Acids[4] | 0.50 | 0.018 | 0.38 | 0.06 | 0.06 | | | |
| IV. Unknown Lipids[5] | 0.87 | 0.012 | 0.58 | 0.09 | 0.19 | | | |
| Sugar | | | | | | | | |
| I. Neutral Sugar[6] | 1.00 | 0.056 | 0.44 | 0.07 | 0.49 | | | |
| II. Amino Sugar[7] | 0.30 | 0.017 | 0.12 | 0.02 | 0.13 | 0.02 | | |
| Media | 21.42 | | 8.79 | 3.04 | 8.99 | 0.96 | | |
| Unknown | 47.66 | | 29.30 | 2.42 | 16.19 | | | |
| Metals | 0.00 | | | | | | | |
| Found | 100 | | 52.55 | 7.71 | 32.76 | 4.32 | 1.82 | 0.84 |

Table 4 (continued)

[1]Protein calculated via total nitrogen minus media and amino sugar nitrogen multiplied by a factor of 6.38; 55,000 was used as an average molecular weight based on the results of Chen and Haines (50).

[2]Sulfolipids based on 2.5 chloro groups average per sulfolipid molecule with an average molecular weight of 595.8.

[3]9.6 molar % sterols in membrane with an average molecular weight of 407.

[4]12.3 molar % fatty acids in membrane with an average molecular weight of 276.

[5]7.8 molar % unknown lipid in membrane with an average molecular weight of 728 (based on diglycosyl diglyceride).

[6]Estimated by GLC (61).

[7]Determined by amino acid analysis (61).

and 457 (diols), respectively. Unknown lipids were averaged as having two stearate chains. Using this approach, the molar per cent chlorosulfolipid, sterols, fatty acids, and unknown lipids in the membrane was found to be 71, 9.6, 12.3 and 7.8, respectively. Using these values, the % weight and μ moles of the sterols, fatty acids and unknown lipids in a milligram of membrane was calculated (Table 4).

The amount of neutral sugars had been estimated on a gas liquid chromatogram which had been calibrated with hexatrimethylsilyl inositol. Amino sugar was determined on an amino acid analyzer (Table 4).

The media component (Table 4) refers to trapped material from the surrounding media during the formation of the vesicles. After isolating the membrane as a pellet, the excess buffer was removed and the pellet dried in vacuo. The difference between the wet weight and the dry weight was taken as a means of determining the amount of surrounding media present. Trapped material had been determined to be at least 14% glucose (49) and based on the composition of the culture media, the remaining trapped components were quantitated.

Elemental quantitation of each membrane component was based on standard compounds.

Those analytical results which could not be accounted for were computed and listed as unknown. The oxygen component of the sample was directly analyzed.

## Amino Acid Analysis

The amino acid composition of the membrane was determined as is shown in Table 5. It should be noted that cysteine and tryptophan were not assayed. Additionally, asparagine and glutamine are shown as aspartic acid and glutamic acid in the table. The quantitation of an amino acid was determined by comparing with a known quantity of that same amino acid.

Table 5

AMINO ACID COMPOSITION OF THE MEMBRANE

| COMPONENT | RESIDUES |
|---|---|
| Lysine | 5.2 |
| Histidine | 1.8 |
| Arginine | 4.3 |
| Aspartic Acid | 10.5 |
| Threonine | 7.0 |
| Serine | 9.6 |
| Glutamic Acid | 10.5 |
| Proline | 4.9 |
| Glycine | 11.9 |
| Alanine | 9.2 |
| Valine | 5.1 |
| Methionine | 1.0 |
| Isoleucine | 4.0 |
| Leucine | 10.0 |
| Tyrosine | 3.7 |
| Phenylalanine | 4.1 |
| Ammonia | 11.3 |

# DISCUSSION

## Isolation of Flagella Components

Two simple and mild isolation procedures have been developed in which a pure flagella membrane preparation in high yields, may be obtained. O. danica is deflagellated by means of a vortex mixer at 4°. This is the mildest deflagellation method yet described. Chlamydomonas flagellar membrane had previously been isolated after addition of a nonionic detergent such as Triton X-100, Nonidet P-40 or Sarkosyl (73). The use of detergent to isolate the membrane may well have affected the quality of the membrane preparation. In the isolation procedure herein described, the membrane was obtained without the introduction of any detergents. The first method is based on suspension of the flagella in a buffer at pH 7.5. O. danica is normally cultured at pH 4.5. The organism does not grow above pH 6. Previously it was shown that resuspension of the flagella in Tris buffer at pH 7.5 would dislodge some of the membrane from the axoneme. This observation was used with vortexing to maximize the release of membrane from the axoneme. The released membrane was then isolated by differential centrifugation.

A second method for the isolation of membrane from the axonemes took advantage of our own observation that 1 mM EDTA at

pH 7.5 (TEM) would solubilize the axonemes of the flagella but not the membrane. It has previously been shown with Tetrahymena pyriformis, that after removal of the membrane which surrounds the cilia by selective solubilization with digitonin, a surface-active glycoside, the remaining axonemes of the cilia were soluble in salt solutions at neutral pH (74, 75). For example, a suspension of digitonin-extracted cilia in 0.6 M KCl became gradually less turbid over a period of several hours as the protein went into solution. Suspension of the demembranated cilia in 0.6 M KI likewise brought almost all the protein into solution, but in this case the action appeared instantaneous. A useful fractionation of the structural protein was obtained by treating the cilia with a chelating agent at very low ionic strength. Suspension of digitonin-extracted cilia in 1 mM Tris-HCl, pH 8.3, 0.1 mM EDTA buffer brought almost all of the ATPase activity (associated with the dynein arms of the axonemes) into solution but only about 30 per cent of the protein. Examination of the insoluble residue in the electron microscope showed that it consisted of the outer fibers alone; the other structural components, including the arms on the outer fibers (Fig. 2) have been almost completely removed.

These various techniques were examined on the flagella of O. danica without the use of digitonin since the increase in pH should have been sufficient to release a significant amount of membrane in order to expose the axonemes to the various solubilizing solutions. However, none of the methods described by Gibbons (74, 75) seemed to cause a significant change in the turbidity of the

flagella suspension.

After a number of trials, TEM was found to solubilize the axonemes almost entirely without digitonin treatment (Fig. 3). The membrane appeared to vesiculize with an average diameter of about 50 to 100 nm and was not associated with any of the axonemes.

It seemed of interest to see whether reconstitution of the fine structure could be obtained by restoring magnesium to the preparation. Various concentrations of magnesium and calcium were used but no significant reconstitution was observed in these experiments.

## Sensitivity of the Membrane Preparation to Various Divalent Cations and EDTA

These studies were undertaken to study EDTA tolerance and metal ion exchange with the natural material. If, indeed, the secondary sulfate groups of the sulfolipid molecule are held in the hydrophobic bilayer by ion pairing to magnesium or calcium, then EDTA would be expected to "dissolve" the membrane by chelating the divalent cation.

In each of the studies conducted, the preparation showed normal trilamellar structure in transmission electron microscopy. In each case there were "normal" vesicles of membranes, generally with diameters of 50-100 nm.

The 0.0045 mM EDTA preparation had vesicles predominantly larger than 100 nm. About half of the membrane in the sample was vesicular. The remainder appeared to be popped vesicles. An

increase in the EDTA concentration to 0.45 mM yielded 68% sheets (popped vesicles). The absence of both metal and EDTA, however, gave results of 82% sheets. With regard to the divalent cations, there was no distinction between 4mM magnesium, 4mM manganese and 10 mM calcium. The latter metal had a different effect at 4mM. Calcium at 10 mM generally fuses vesicles of phospholipid bilayers especially when the lipids are anionic.

That the membrane maintained its basic structure in the presence of EDTA and 10 mM calcium are important points. They imply a remarkable insensitivity to divalent cations and suggest that they are not involved in maintaining the integrity of the bilayers. The greater stability of the membrane in EDTA as compared to Tris/mercaptoethanol alone may be ascribed to the greater ionic strength of the EDTA solution. These observations suggest that there are no divalent cations in the bilayer which has been confirmed by the elemental analysis discussed in a subsequent section.

## Distribution of Anionic Sites on the Plasma and Flagellar Membrane

The distribution of negative sites on the cell surface has been studied with the use of colloidal iron as the visual marker (80—82). However, Danon et al. (67) introduced the use of polycationic ferritin (PCF) for localizing anionic sites. This electron-dense probe is now preferred because it is possible to treat live cells at physiological pH, thus avoiding the introduction of fixation.

In this study, the distribution of anionic sites on the surface of the plasma and flagellar membrane was examined with the use of PCF. PCF binding may be correlated to chlorosulfatide accessibility to the ligand since the flagella and flagellar membrane are devoid of sialic acid residues (61).

The data suggest that the chlorosulfatides are partially buried under periferal proteins, glycoproteins, polysaccharides or a different polymeric coat on the cell surface causing low density of PCF binding. However, modification of this surface causes a breakdown of the polymeric coating and provides the PCF with access to the anionic membrane. Accordingly, the membrane surface becomes heavily labeled Vesicles secreted by the cell appear to have released their coating and show a high density of PCF binding. The process may be observed in Fig. 7 where a small projection of the cell surface exhibited higher PCF binding than the rest of the surface. Presumably this projection has broken through the cell surface coat and will pinch off to become an extracellular vesicle. These observations are consistent with PCF binding to the isolated flagellar membrane. Isolated vesicles show high density ligand binding although some exhibit no PCF interactions. Those vesicles that do bind PCF appear to have either regions of high density binding or the scattered binding typical of whole cell membrane. Presumably the low density binding regions still contain the surface coat, thereby not allowing accessibility of the PCF to the anionic membrane lipids.

Kahan et al. (83) have used the cationic dye, ruthenium

red, in order to demonstrate a surface coat. The stain was much more apparent on rough-surface organisms, i.e., those secreting membrane vesicles, than it was on cell membranes with smooth surfaces. The staining of the cell surface with ruthenium red (a small molecule) indicates a loose polymeric cell coat which does not allow larger molecules (such as PCF) to penetrate--quite possibly a physiological necessity.

In the case of the freshwater flagellates and ciliates one might expect that the lipid composition of the flagellar and ciliary membranes would necessarily be of unusual structures. This is so because such lipids would be exposed on their outside surfaces in nature to lipolytic enzymes from other organisms which would likely destroy the membrane. Most bacteria and other microbes that have been examined are well protected by a protein, polysaccharide, glycoprotein or other polymeric coat which would protect them from such enzymatic attack. In O. danica, the secretion of vesicles made of sulfolipid devoid of the coat, could be a superb defense mechanism since the lipids may be considered as denaturing detergents. One might speculate further that if such vesicles contained hydrolytic enzymes--themselves resistant to the detergents--the system would be potent indeed.

## Freeze Cleavage of Flagellar Membrane

The most persuasive evidence for the bilayer theory of membrane structure is that obtained by freeze-fracture electron micrographs.

The investigations of Branton (21) have convincingly demonstrated a "cleavage plane" down the center of a variety of natural membranes.

The stability of this model is dependent upon the structure of the polar lipids which are present in natural membranes. To date, all polar lipids that have been found in membranes, except for the chlorosulfolipids, consist of hydrocarbon chains which terminate as a methyl group at one end and a polar hydrophilic group at the other. The entire length of the lipid molecule from the methyl group to the hydrophilic end is hydrophobic. The sulfolipids do not conform to this generalized structure of membrane lipids and presumably should not be suitable for the formation of a bilayer. However, with the establishment of the sulfolipids

in the membrane (and indeed 90% of the polar lipids in the membrane), it was important to demonstrate a cleavage plane. Figure 12 is, therefore, of interest as it shows a freeze fracture of the isolated flagellar membrane vesicles. The freeze fracture clearly shows the flagellar membrane capable of fracturing down a "cleavage plane."

The smooth quality of most of the fracture faces is not too surprising since the flagellar membrane would not be expected to maintain a transport function. Such a smooth flagellar membrane freeze fracture was observed by Bergman et al. (84) who observed occasional rows of "bumps" extending from the base to the tip of Chlamydomonas reinhardii flagella.

## Elemental Analysis of Flagellar Membrane

The divalent cation composition of membrane preparations was studied in order to establish the molar equivalence of calcium and magnesium to that of sulfate ester in the membrane. This was necessary as the secondary sulfate groups may have been held in the hydrophobic bilayer by ion pairing to magnesium or calcium (which would have been the most probable candidates) shielding the charges of the two secondary sulfates and thereby permitting overall hydrophobicity. In order to draw useful conclusions, a large batch of pure membrane was isolated in 4 mM manganous acetate in which the membrane was stable (see Table 2). The membrane very likely has binding sites for these divalent cations; for example, Fay and Witman (86) have demonstrated the presence of a calcium activated ATPase from Chlamydomonas flagellar membrane. It would not have been surprising therefore to find divalent cations bound to protein in O. danica's flagellar membrane. A major problem in quantitating the flagellar membrane components in this experiment was the occurrence of trapped material from the flagellar cytosol during the formation of the vesicles. The results showed that either magnesium and calcium were present in sufficient concentration to bind to the secondary sulfate groups on a one to two basis if they were in the interior of the membrane.

A repeat of this experiment was conducted when the flagellar membrane was isolated in EDTA buffer (TEM). No metals were detected (Table 4).

Careful quantitation of each membrane component have lead to the following conclusions:

1. All of the nitrogen is accounted for by either protein or amino sugar. Therefore, an organic nitrogen cation that could shield a charged sulfate in a hydrophobic region, such as a polymeric glycopolymer that have been isolated from cell membrane and envelope systems, is discounted as is a small divalent organic cation.

2. An unknown component is present in the membrane which is 61.2%C, 5.1% H and 33.7% O. This could indeed be the membrane coat suggested by Figures 7 and 8.

Membrane Amino Acid Analysis

The absence of appropriate amounts of divalent cations and organic nitrogen molecules suggest membrane protein as supplying the necessary counterion to the secondary sulfate groups. In

this case an arginine or lysine residue on an "integral" (16) protein would be likely. As determined by amino acid analysis (Table 5) this appears not to be the case as it would require high concentrations of the basic amino acids. The basic amino acids are relatively low in the composition. The protein composition suggests, on the contrary, that the proteins are acidic.

## A Model for the Structure of the Flagellar Membrane of O. danica

When the lipid composition of the flagellar membrane was first examined, free fatty acids (12.3 molar %) were found as an important component of the membrane (49). It was immediately suspected that these substances appeared as a result of the activity of a lipase in the extract. In an attempt to exclude the possibility of lipase activity, (1 - $^{14}C$) - acetate labeled membrane was spotted directly on a dry TLC plate (which had previously been heated) so that the relative amount of free fatty acids could be compared to those in the lipid extract. The relative amount of fatty acids in each extract was identical. It was conceivable that the lipase was nonetheless active and therefore the source of the free fatty acids was not entirely clear. Should they exist in an anionic membrane, they would add anions to the anionic surface too close to the hydrophobic region and further destabilize the membrane by surface charge repulsion.

The following discussion, however, sheds a different light on the subject. The free fatty acids in the membrane may indeed be

the source of the stability of the bilayer. The pK of long chain fatty acids is 4.76 when the ionic strength of the solution is 0.1 (87). Smith and Tanford explored the distribution of fatty acids in organic (heptane) vs. aqueous phases at different pH's. This system had first been explored by Peters (88) who found that the pKa of fatty acids at the benzene-water interface was three pH units above that in the bulk aqueous solution. Thatanionic surfaces have high acidity in the immediate vicinity of the surface was first explained by Gouy (89). The counter ion (a cation in the case of an anionic surface) is in the bulk solution and a high concentration of anions at a fixed surface ionizes the water so that protons remain near the negative surface and maintain local neutrality. A calculation by Davies and Rideal (90) shows that a negatively charged surface of 200 mV potential attracts enough protons to lower the surface pH by 3 pH units.

Peters (88) showed that the fall in interfacial tension in a benzene-water system containing fatty acids commenced on the alkaline side of pH 4.5 and followed a straight line to pH 9. He estimated that the pH at the interface was about three units below that of the bulk medium. Most important, however, is that he was titrating the fatty acid over this range and that the effect on the surface tension was linear. Smith and Tanford (87) have shown that aggregates higher than dimers are formed in the aqueous phase as the pH is raised. This would account for such a linear change.

Ochromonas danica is cultured best at a pH of about 4.5. Although the organism survives at pH's above 7 its growth is negligible. The lipid composition shows that approximately 70 molar per cent of the lipid in its flagellar membrane is the mixture of chlorosulfatides and that nearly 25 molar percent is a mixture of sterols and free fatty acids.

Since acetate-$^{14}C$ labeling experiments (49) showed the chlorosulfatides, the free fatty acids, and the sterols together constitute over 90 mole per cent of the membrane it must be assumed that these substances are on both sides of the bilayer.

A novel role for the fatty acids of the flagellar membrane on the molecular level may now be suggested. This discussion will defer the problem of the secondary sulfates which will be discussed later and will assume that the chloroalkyl disulfates are strictly alkyl sulfates.

The pK of an alkyl sulfate is below two. How far below two is not known as the first pK of sulfuric acid is 1.9 (91). It might be inserted here that these equilibria refer only to aqueous solutions. The variations that may occur in a non-aqueous environment are illustrated by the fact that esters of fatty acids can be formed at equilibrium in a non-aqueous phase which is in contact with neutral buffer (92). This despite the fact that the equilibrium in the buffer solution is far toward hydrolysis. This observation also influences consideration of the alkyl sulfates in the membrane (including especially sulfate esters in the hydro-

phobic interior of the bilayer).

If the pH of the bulk solution is 4.5 to 5.0, the pH at the surface of the anionic membrane can be estimated to be 1.5 to 2.0 (another way of stating this is that the anions are solvated and that their pK's are now three units higher). At this pH all fatty acids are protonated. In order to approach the pK of the fatty acids where the acids would be 50% protonated it would be necessary to raise the pH of the bulk medium to 7.7, a condition most undesirable for the organism. We have assumed until now that the sulfate esters are charged but what is now very interesting is that the pK of the sulfate esters may themselves be in the range of the pH at the surface of the membrane.

Gebicki and Hicks (93) made vesicles of oleic acid buffered at pH 8 - 9 which retained glucose until disrupted by Triton X - 100. These studies were later expanded (97) to include linoleic acid and a preparation of liposomes (probably multilamellar) was obtained which contained water. The liposomes were impermeable to glucose and stable at pH 8. Although these investigators undoubtedly had multilamellar vesicles their glucose retention and osmotic swelling studies (in addition to the electron microscope studies) have together established that there is a bilayer formed. This is rather impressive if one took at face value that the entire surface of the liposome would be considered anionic at pH 8. However, is is now known that at the anionic surface the pH is closer to 5. Another interesting feature of the work of Gebicki and Hicks is that they were unable to form these vesicles of saturated

fatty acids which is why they called them ufasomes (unsaturated fatty acids liposomes). Furthermore, the linoleic acid (18:2) derived liposomes behaved as though they had greater stability than those made from oleic acid (18:1).

Rosano, et. al., (94) and Feinstein and Rosano (95) have titrated potassium myristate solutions (50 mM) with acid and found that upon titration a precipitate formed which had a 1:1 acid to soap ratio. This precipitate was formed as the micelles were neutralized and not until all of the micelles were neutralized did the non-micellar fatty acids become neutralized to form a pure fatty acid precipitate. These observations are especially important since the pH at which the 1:1 acid-soap mixture (at an anionic surface) buffered the solution at pH 8.3 and the free fatty acid anions were neutralized at 4.5. Additionally the 1:1 soap to fatty acid precipitate points up the special stability of the $RCOO^-$ complex with RCOOH. The remarkable stability of this complex is not new. Smith and Tanford (87) state that "hydrogen bonds between $RCOO^-$ and RCOOH are extraordinarily stable when carboxyl groups are attached to long alkyl chains." The recognition of this special stability goes back to the work of Westheimer and Benfey (96) who studied the pKa's of dicarboxylic acids containing hydrocarbon groups. The presence of the alkyl group on the molecule was shown to participate in stabilizing the $HA_2^-$ anion. It appears, therefore, that this is the basis of the stability of both the fatty acid liposomes of Gebicki and Hicks (93) and of the crystalline state of Feinstein and Rosano (95).

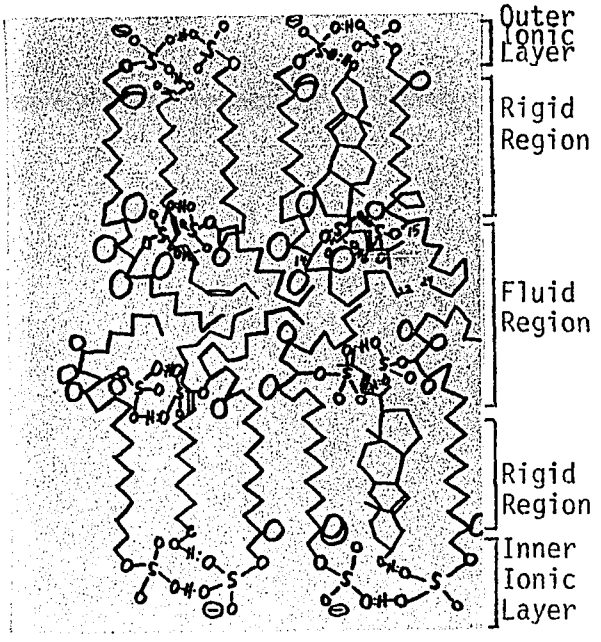
In view of the above it is not, therefore, surprising that Hargreaves and Deamer (98) have obtained liposomes (including some unilamellar vesicles) from dodecanoate and dodecanol mixtures and from dodecyl sulfate and dodecanol mixtures, and oleate-cholesterol mixtures. They also obtained vesicles from all of the pure saturated fatty acids they tested (at 50 mM concentration), provided they were above the transition temperature (Tm). Indeed they also obtained sucrose impermeable vesicles from octadecyl sulfate and octadecyl phosphate. This work especially notes the necessity of using temperature above the Tm of the lipids of the bilayer and some of the procedures for the formation of vesicles are conducted as high as 60°. This explains the requirement in the Gebicki and Hicks studies for unsaturated fatty acids. A second important observation of Hargreaves and Deamer (98) is that either one must conduct the liposome formation about 3 pH units above the pK of the anion (in addition to the Tm requirement) or there must be "spacer" molecules in the plane of the bilayer to separate the charged head groups.

The implication of the latter finding is that the fatty acids may serve as "spacer" molecules in fatty acid liposomes when the polyanionic surface is three pH units below the pH of the bulk medium if the bulk medium is three pH units above the pK. Under these conditions 50 per cent of the fatty acid molecules are protonated and 50 per cent are anionic. The protonated fatty acids are being utilized as "spacer" molecules for the anions and the surface is stable.

The alternative is a long-chain alcohol or a sterol. Liposomes

containing such molecules are stable at pH's in excess of the 3 units above the pK of the anion.

The implications of this information to the flagellar membrane of O. danica is that the membrane lipids may be considered as producing a polyanionic surface. The sterols and the fatty acids may both be considered "spacer" molecules (Fig. 13). This is so because the pH of the bulk medium is 4.5 which means that the surface pH is about 1.5. Thus all of the fatty acids will be protonated and will participate as "spacers." The chlorosulfatides, on the other hand, will behave largely as anions. Although the pK of the sulfatides is not known it is probably in the range of 1.5 (91). A significant portion of these molecules may be in the protonated state which would convert them to "spacers" as well.




Sulfate groups are at or near the pK. Some are charged creating the anionic surface. Others are protonated and serve as "spaces" separating the anions which would otherwise be repulsive. Sulfate groups in the hydrophobic region are di-protonated dimers.

Fatty acids are protonated and--like sterols--also serve as hydrogen bonding "spacers."

Chloro groups are the size of methyl groups and facilitate the fluidity of the fluid region.

Fig. 13. Proposed model for the arrangement of chlorosulfolipid in the flagellar membrane of O. danica.

In view of the above discussion it is now clear that one might suspect fatty acids and some sulfatide to exist on the surface of the membrane in the protonated form. Several lines of evidence suggest that the sulfatides are not present in the membrane bilayers as divalent cation salts. The first is that the membrane is stable in the presence of 1 mM EDTA. Secondly, elemental analysis of this membrane revealed no metal cation present. Additionally, results presented here shows the presence of no organic nitrogen compound capable of shielding the secondary sulfate in the hydrophobic region of the membrane.

The secondary sulfate esters are suspected to be deep in the bilayer in the protonated form (Fig. 13). Such a suggestion has many attractive features. The first is that the secondary sulfates (which are tetrahedrons) are held together with two hydrogen bonds if each is protonated.

The function of the chloro groups on the chlorosulfolipids in this hypothetical discussion of an extremely acid bilayer is as follows:

It is apparent that the chloro groups which are sterically the size of methyl groups may enhance the liquidity of the membrane. This is analogous to the cis double bonds of the polyunsaturated fatty acids. It is important to note here that the data of Gebicki and Hicks (97) showed that only unsaturated fatty acids were capable of forming fatty acid bilayers--so much so that these investigators called them "ufasomes." On the other hand, Hargreaves and Deamer (98) were able to extend their work to saturated fatty acids by raising the temperature and by shortening the chain length so that the liposomes were above their transition temperature. Chen et al. (49) have noted that the O. danica flagellar membrane fatty acids are either short or polyunsaturated.

It is interesting to speculate that at a pH of about 7 in prebiotic earth, the first bilayer was a bilayer of fatty acids just 3 pH units above the pK. It may well be that O. danica and the other organisms that contain these chlorosulfatides exemplify an early fork in evolution in which the fatty acid bilayer never achieved what is probably the evolutionary advantages of the phospholipid bilayer.

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