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City University of New York PH.D. 1982

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STUDIES ON THE EXCRETED ALKYL SULFATE MEMBRANE OF *OCHROMONAS DANICA*

by

EZZAT EL-MARAGHY

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.

1982

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

4/22/82
Date

Chairman of Examining Committee

4-30-82
Date

Executive Officer

Supervisory Committee

ABSTRACT

The isolation and purification of the extracellular vesicles [ECV] of the phytoflagellate, Ochromonas danica, by two procedures is described. The procedures were simple, rapid and produced a pure membrane preparation. The isolated ECV was free of other cellular organelles as shown by electron microscopy. The membrane preparation consisted of uniformly sized vesicles (preparation I), with a trilamellar pattern. The preparation was analyzed for its lipid, protein and carbohydrate composition. The lipids of the ECV were nearly identical to those of the flagella membrane. The polar lipid did not contain phospholipids and were exclusively the chlorosulfolipids. These were about 70% of the total lipids, the remainder was non-polar lipids.

These sulfolipids have been previously characterized as 1,14-docoanediol-1,14-disulfate and 1,15-tetracosanediol-1,15-disulfate containing up to six chloro groups replacing hydrogen on the otherwise saturated hydrocarbon chain.

The non-polar lipids (total 22%) were dominated by a large amount of free fatty acid (∿14%), and sterols (∿7%).

The ECV were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and found to contain 4 major protein components. These had apparent M_R of 97,000, 85,000, 47,000 and 19,000. In addition one minor protein of apparent molecular weight of 110,000 dalton was found. All protein components were shown by periodic-acid-Schiff reagent to be glycoproteins.

The analyses showed that carbohydrate dominates the ECV (∿ 33%).

The major neutral sugar is mannose.

The charge distribution on the surface of the ECV was examined by the use of the alkylated pH indicators 4-heptadecyl-p-amino-coumarin and 4-undecyl-p-hydroxycoumarin. Using these indicators it was possible to measure the pH within 1.0 nm the of membrane surface. The results show that the membrane surface has a negative charge typical of model alkyl sulfate membranes, providing direct evidence that the alkyl disulfates behave as membrane polar lipids.

A study of the capacity of alkaline pH and sonication to dissolve this unique membrane indicates that the ECV alkyl disulfate membrane is stabilized by a high proton concentration.

Bovine serum albumin (BSA) was successfully used to remove some of the free fatty acids verifying their presence in the natural ECV membrane. The formation of multilamellar myelin-like membranes provoked by BSA treatment was demonstrated by electron microscopy.

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Finally, I thank my parents for their prayers and their support and to my wife Nadia for her love and patience throughout these years and to my children Maggie and Michael.

It is to them this work is dedicated.

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INTRODUCTION

It is widely believed that biological membranes are made primarily of phospholipids and glycolipids. These polar membrane lipids have two aliphatic chains for each polar head-group. In the universal structure of the polar component (ionic or glyco-) of these lipids, there is a glycerol moiety (or its equivalent, e.g., the first three carbons of sphingosine in the sphingo lipids). The conformation of these lipids in bilayers, based on X-ray and NMR data, places the ionic groups in the aqueous phase and the glycerol moiety below them (toward the hydrocarbon chains). These general features of membrane lipid structure and conformation are so widespread that it would be most unusual to find a membrane that is made of lipids the structures of which violate these precepts.

Phytoflagellates are a group of photosynthesizing protozoa of widely varying morphology, biochemistry and physiology. They live in a variety of environments, both fresh water and marine (1). *Ochromonas* is a typical fresh-water phytoflagellate; it has attributes of animal (ingesting, motility) and plants (photosynthesis) (2,3,4). The flagella of *Ochromonas danica* consist of bundles of microtubules, arrayed in the typical 9 + 2 pattern (axoneme), surrounded by a membrane. Attached to the membrane are extraflagellar hairs, called mastigonemes. Fig. 1 represents a sketch of the *O. danica*. The mastigonemes of *O. danica* are of two structural types, fibrous and tubular. The tubular mastigonemes are arranged in rows on opposite sides of the flagella along the entire shaft (5,6). *O. danica* moves by means of a planar sine wave in the long anteriorly-directed

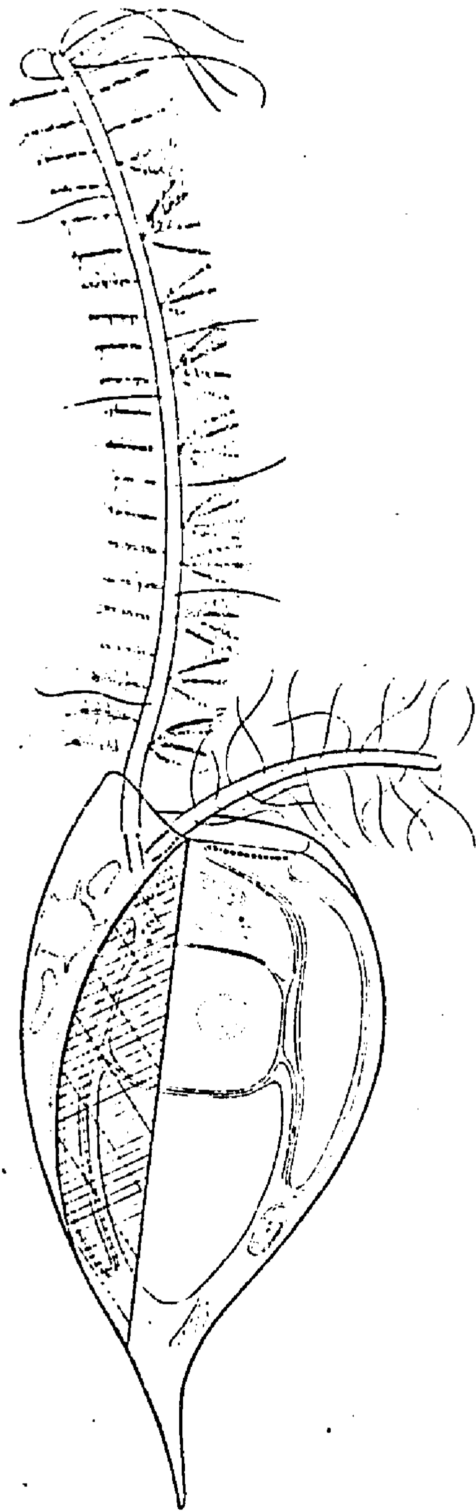


Figure 1.

Figure 1. Reconstructed drawing of the phytoflagellate, *Ochromonas danica* based on electron micrographic studies. The figure illustrates the two flagella of the organism. It is drawn approximately to scale. The flagella have hairlike projections (mastigonemes) which are somewhat oversized in order to illustrate details. Taken from G.B. Bouck (J. Cell Biol. 50, 362 (1971) with permission.

flagellum. The wave starts at the basal end of the flagellum and progresses distally. It has been proposed that one function of these mastigonemes is to provide a roughness of surface, which is responsible for movement in a direction opposite to that hydrodynamically expected from a naked flagellum (7).

O. danica contains a substantial quantity of unusual sulfolipids. These compounds consist of two series of disulfates (Fig. 2). They are docosane-1,14-disulfate, and tetracosane-1,15-disulfate each associated with a group of polychloro derivatives with from 1 to 6 chloro groups replacing hydrogen atoms on the otherwise saturated alkyl chain (8-15). These alkyl sulfates are the only known membrane lipid molecules which contain two polar groups at both ends of the molecule. A wide variety of sulfolipids have been found in nature including in mammalian tissues. Generally sulfolipids are found in membranes rich in acidic lipids.

In particular sulfolipids have been identified with flagellar (but not ciliary) membranes. A prime example is the sperm which in all known species contains sulfolipids. The first to be characterized was sulfogalactosyl diglyceride (SGG) in rat (17). SGG has since been found in the mature testes of many or most mammals (18). It is synthesized by the germinal cells during one discrete stage in spermatogenic differentiation, probably in leptotene spermatocytes (17). SGG is sometimes used as a differentiation marker in spermatogenesis and appears coincident with germ cell transition from the basal to adlumenal compartments of the seminiferous tubule. The appearance of SGG is thus consistent with

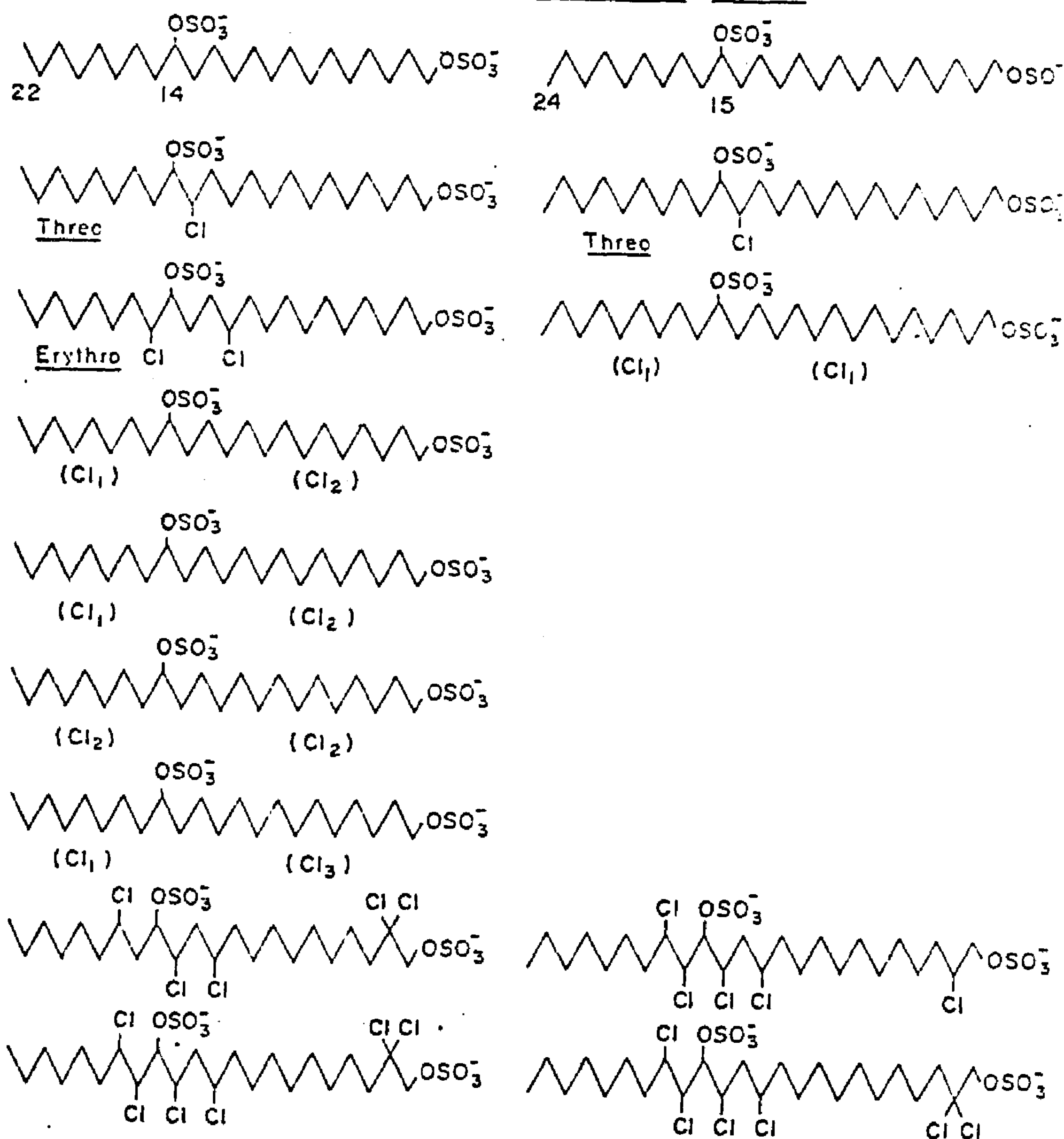


Figure 2. The structures of chlorosulfatides.

the appearance of flagella. It is also interesting that sulfotransferase appears in the ontology of testis before any SGG can be detected. This suggests that the control of SGG synthesis may lie in the availability of its precursor galactosyl diglyceride (GDG), since GDG appears in the developing testis immediately before SGG (19) and, SGG, once made, does not turn over and is lost from the testis only by the maturation and departure of germinal cells. Recent work (20) has localized SGG in the plasma membranes of testicular germinal cells. This suggests that it must be present in the flagellar membrane since these membranes are continuous.

Liem and Laur in 1976 (21) have found four new aliphatic sulfates in three fucacea (brown algae) from the coast of Britany P. canaliculate (L), Fucus vesiculosus (L), and F. 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-1,18-diol-1,18-disulfate. Other than the alkyl sulfates of brown algae the only natural alkyl sulfates known are those of O. danica which have since been found in some 30 species of fresh water algae (23).

They are absent from 8 species of marine algae. Mercer and Davies (22,24) had earlier 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. In most organisms these are present in small amounts ($< 2.0\%$). In a few chrysophytes they are present in significant quantities. In Ochromonas danica the chloro-

sulfolipids are a greater fraction of the total lipids than any other known organism (>14%).

Over 90% of the polar lipids of the flagellar membrane consists of these chlorosulfolipids (25). The compounds are truly unique membrane lipids. In addition to the chlorosulfolipids, the flagellar membrane, which is approximately 8 nm thick in electron micrographs is never found associated with a wall or a second membrane. 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 (for a fresh water organism) between the cytoplasm and outside medium. It shows no osmolality when the organism is suspended in deionized water. Most cells that do not burst under these 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 be exposed in nature to lipolytic enzymes from other organisms which would be expected to destroy the membrane. Most microbes are protected by a protein, polysaccharide, glycoprotein or other polymeric coat which would protect them from such enzymatic hydrolysis. There are only three organisms that lack such enzymatic protection in which the lipids have been characterized, namely, the cilia of _Tetrahymena pyriformis_ (26-28) and _Paramecium tetraurelia_ (29) and the flagella of _O. danica_ (30). Each have turned out to contain primarily lipids with exotic structure.

The surface of the flagellar membrane of O. danica contains glycoproteins (5), (31). The sugar composition of the membrane is dominated by rhamnose (32). The absence of phospholipid-like structures from the plasma membrane would suggest that the organism has a radically different microenvironment for its membrane proteins within which these proteins engage in fundamentally the same transport activities and dynamics as those of typical phospholipid plasma membrane.

The alkyl sulfate membrane has the following specific differences (due to the structures of its components) from the ordinary membranes:

1. It contains single chain alkyl sulfates as the sole polar lipids. These molecules lack a glycerol domain below the anion of the headgroup.
2. They have twice the density of headgroup anions at the surface as does the typical dialkyl chain phospholipids.
3. A secondary sulfate is present on each alkyl sulfate chain at position 14 or 15, although the carbon number is different, models suggest that this bulky group if present in the hydrocarbon is at the same level as the 9 double bonds of phospholipids that contain oleic acid. This is also the location of the termination of the fused ring of sterols (such as cholesterol) and the beginning of the sterol branched side chain (33).
4. Although the chains lack double bonds there are up to six chloro groups replacing hydrogens on the chain. These are clustered around the two sulfates. They may make the sulfate

more acidic (by induction); they add bulk (a chloro is the steric size of a methyl group) to the chain at selected sites; and they change the "solvent properties" of the bilayer at the cross-sectional height of the bilayer. Thus the presence of the chloro groups effectively converts the "solvent" of the bilayer from alkane (saturated chain) to halocarbon (e.g. chloroform).

5. The fatty acids in the bilayer are largely free fatty acids in contrast to those of traditional bilayers.

There are certain other membranes (34,35) where free fatty acids have been reported.

Due to the totally anionic sulfate groups at the surface of its membrane and since these single-chain lipids contain a secondary sulfate on the 14 or 15 position of the chain a reasonable question is, "How can a natural membrane be made of such repulsive detergent and soap molecules?" Studies (36) were initiated to seek a counterion of either the metallic (Ca^{++}, Mg^{++}, etc.), organic (R_3NH^+), or protein type. None was found and it was concluded that the proton served as the counterion. Two different kinds of studies have been used (37,38) to examine acidity at the membrane: 1, ^{13}C-NMR of carboxyl-labelled fatty acid (lauric acid) in which the protonation state of the fatty acid could be observed directly by the chemical shift of the probe, and 2, fluoroscent alkylated pH indicators developed by Fromherz and Fernandez (39) to examine the hydrogen ion concentration in the vicinity (within 1.0 nm) of the membrane surface.

It is not surprising to find that O. danica has an acid membrane.

Many natural membranes are constituted solely of anionic lipid headgroups although many membranes contain uncharged (glyco-) lipids as well. Membranes made only of anionic lipids include the membranes of all archaebacteria (40,41), of all photosynthetic membranes (including chloroplasts), and of many bacteria that are neither archaebacteria nor photosynthetic. Since the earliest organisms in evolution are believed to be archaebacteria or photosynthetic (41), the acid membrane may be important in the origin of life. Anionic lipids have also been shown to be critical for the survival of living cells that contain both anionic and zwitterionic lipids. This has been directly shown by studies on mutants of E. coli (42) and on mammalian cell lines (43).

Liposomes constituted solely of fatty acids and containing entrapped aqueous compartments were first described by Gebicki and Hicks (44). Because they could only make them by using unsaturated fatty acids they believed this was an absolute requirement for fatty acid liposome stability. Hargreaves and Deamer (45) expanded these studies and demonstrated that the unsaturated requirements in the hydrocarbon domain was due to the fact that stable liposomes could only be formed above the transition temperature of the bilayer. Thus entrapping liposomes could be made of saturated fatty acids of short chain length (C_{14}) at room temperature or of long chain lengths (C_{18}) at elevated temperatures. That these liposomes require a transition temperature and that preparations of them contain unilamellar (in addition to the predominantly oligo-lamellar) entrapping bilayers, suggests that they have qualities that are similar to biological mem-

branes. They differ most significantly from biological membranes in that they are maximally stable in a relatively narrow pH range, pH 7 to pH 9.6.

The existence of fatty acid acid-anions has been known to lipid chemists for many years. A titration of micellar soap solutions by Rosano (46) produced a precipitate that analyzed: two fatty acid chains to one potassium. Smith and Tanford (47) stated that "hydrogen bonds between COO^- and COOH are extra-ordinarily stable when carboxyl groups are attached to long alkyl chains". It is reasonable to assume that the fatty acid liposomes that contain both protonated and unprotonated carboxylates will form acid-anions stabilized by charge distribution. For a membrane surface this also implies reduced charge density. The unusual pH properties of anionic surfaces including particularly the low surface pH at low or intermediate ionic strengths is now widely recognized in the membrane field. The latter has been directly measured at bilayer and micelle surfaces by lipoid pH indicators (39). This low surface pH is based on the capacity of the polyanionic surface to sequester cations within 1.0 nm of the surface (48). At high ionic strength such cations are predominantly the cation of the salt. At low ionic strength the cation is predominantly the hydronium ion due to the ionization of water. Knowledge of the charge density of the fixed surface anions and the ionic strength permits a direct calculation of the pH at a given surface (48). The surface pH may be as much as 3.0 units below that of the bulk pH.

Although phosphatidyl serine (PS), gangliosides and certain

other membrane lipids (49) contain carboxylates most membrane lipid anions are phosphate or sulfate. In the case of phosphate the anion is generally the diester anion. Like sulfate (which is always a monoester) the phosphate diester anion has an unusually low pK (below 4). It is therefore generally presumed that each phosphate in a membrane has a single negative charge above the observed pK of a phospholipid. Because of the unusual surface pH at polyanionic surfaces this may not be true for membranes made of anionic phospholipids. The same statement applies to the chlorosulfolipid membrane of O. danica in which alkyl sulfates at the surface may occur as acid-anion dimers. If such a concentration of protons exists at the surface, then it is not altogether surprising to have fully protonated sulfate buried in the hydrophobic domain. A protonated sulfate in the hydrocarbon region becomes comprehensible because such a sulfate will be expected to form a dimer with a neighboring sulfate (especially when they are the predominant polar lipid). In this view, hydrogen bonding involving very acidic protons may play an important role in the stablization of O. danica's membrane.

Attempts to isolate the plasma membrane of O. danica have been unsuccessful, possibly because these detergents denature enzymes (10) and perhaps even organelle membranes during cell fragmentation so that the plasma membrane preparations obtained during these attempts were heavily contaminated. In an attempt to establish whether or not the entire plasma membrane is "chlorosulfolipid membrane" rather than a phospholipid membrane as found in all other eukaryotes, we have taken advantage of an unusual biological property of the organism:

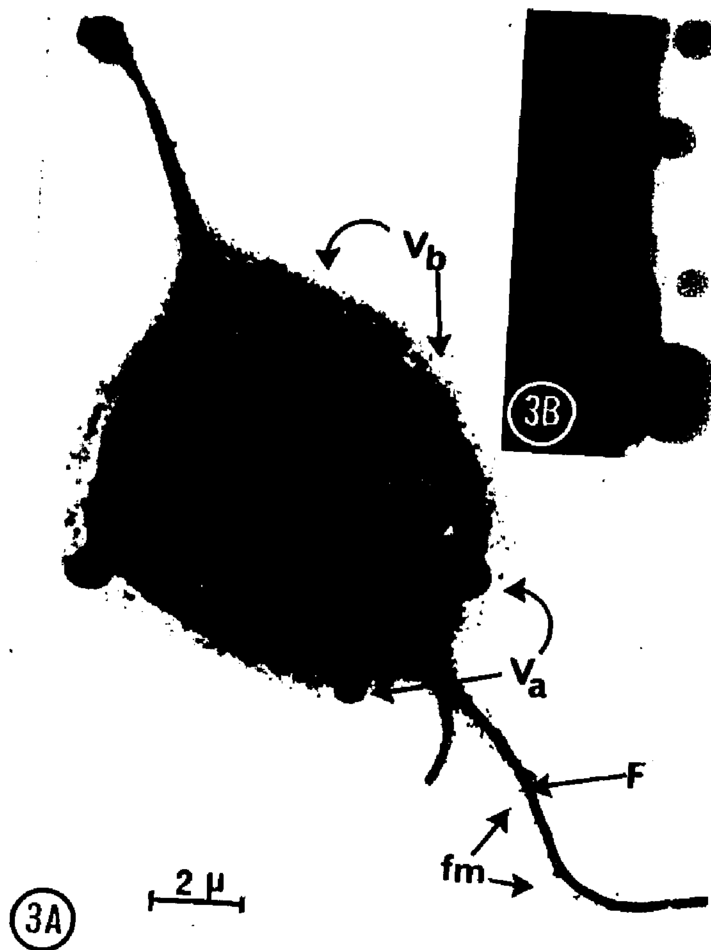


Figure 3.

(A) Negative stain of <u>O. danica</u> cell showing the extrusion extracellular vesicles (X 11,200)

F: flagellum

F_m: filamentous mastigonemes

V_a: blebbing vesicles

V_b: extracellular vesicles

(B) Enlargement of the surface of negatively stained showing vesicles at or near cell surface (X 47,900).

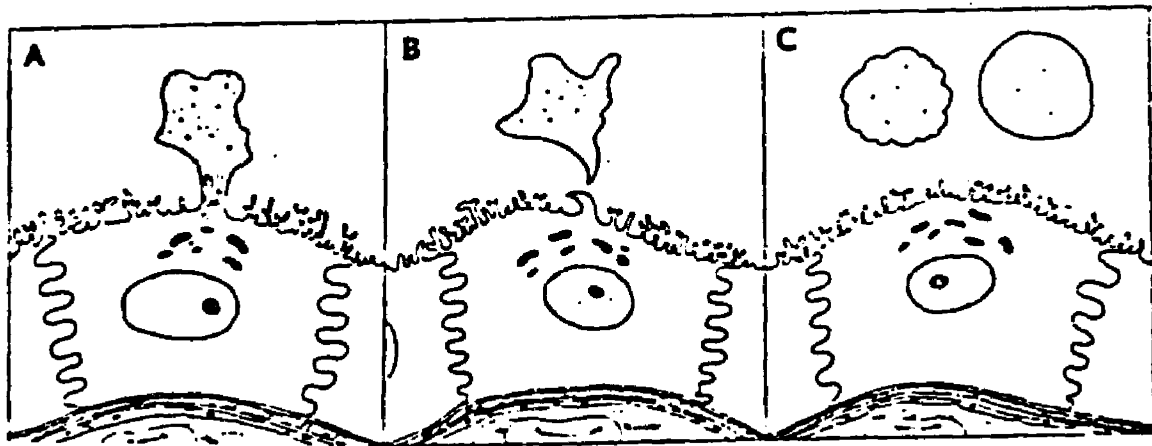


Figure 4. Diagrammatic Presentation of Blebbing in the Choroid Plexus. Taken from Agnew et al. (57) with Permission.

its extrusion of extracellular vesicles during the log phase of growth (50,51). The exocytosis described in these studies occurs from the entire plasma membrane including the flagellar membrane (Figure 3) Unlike other unicellular organisms that grow in fresh water, O. danica, as mentioned above, lacks an external wall or protective coating that is visible in the electron microscope. This unique feature of the organism implies that budding and exocytosis of this membrane yields vesicles of plasma membrane in the culture medium.

Exocytosis involves the formation of blebs at the membrane surface followed by a pinching-off process. Such bleb formation with the consequent separation of the bleb from its cell of origin is shown diagrammatically in Figure 4. Exocytosis is often a characteristic of gram negative organisms, where it is restricted to the outer membrane and it therefore characteristically results in the vesicles' containing the microbe's antigen(s) (52-56), furthermore a variety of degradative enzymes have been shown to be present in the cell envelope of gram negative bacteria, both in the periplasmic space (58,59) and at the cell surface (59). Alkaline phosphatase is typical of these enzymes. It has been shown to be produced in the cytoplasm and secreted into the periplasmic space in the form of inactive subunits (60) which will form an enzymatically active dimer in the presence of specific cations and if certain physical conditions in this area of the cell wall are favorable (54). Exocytosis sometimes implies the fusion of a secretory vesicle with the cell membrane and the temporary formation of a membrane continum. Many cells perform

exocytosis exclusively at certain regions, e.g., the apical pole. The arrangement of microtubules has been proposed to guide secretory vesicles to their potential fusion site (61), and could thus account for some spatial specificity of exocytosis. Membrane behavior during exocytosis has been studied by freeze-fracturing in mast cells (62).

Blebbing is a well-known cell phenomenon in which pleomorphic cytoplasmic protrusions extrude from the cell surface and then retract. Such structures have been observed for many years (63,65) largely in cultured cells. They have been described in connection with cellular locomotion (65,66). The principle motile force for cellular locomotion associated with blebbing is generated by a system of cytoplasmic, 6-8 nm microfilaments which are known to contain actin or actin-like proteins. Schenk and Konrad (67) claim that these microfilaments are responsible for the pinching-off process of blebs. After the pinching-off process certain morphological changes occur in the blebs. Blebs have been isolated in rabbit lung macrophages and were analyzed biochemically by Davies and Stossel (68). They conclude that blebs were metabolically viable sacs of plasmalemma containing contractile proteins and cytoplasmic enzymes. Harris (69) claimed that blebbing in normal cells in vitro as well as in vivo is a reversible process, the blebs expand rapidly from the cell surface but retract somewhat slower under his experimental conditions (70).

The formation and detachment of cytoplasmic bodies (blebs) from lymphocytes has been reviewed by Shields (71). Bleb formation is not restricted to lymphocytes and is known to occur in other cell

types such as erythrocyte plasma cells (72) and megakaryocytes (73). In the latter it resulted in the formation of platelets. In lymphocytes, a row of small vesicles is sometimes observed at the base of these cytoplasmic blebs, and it appears that they form prior to the shedding of the blebs (72). The fate and function of the blebs shed from lymphocytes is still obscure. Shields (71) suggested that lymphocytes have a trophic function that is accomplished by the disintegration of these blebs, thereby contributing to the formation of some of the plasma components. Others have suggested that lymphocytes secrete antibodies by blebs (74). The formation of blebs from hyperplastic thyroid gland has been reported (75). The findings imply that blebs are not restricted to cultured cells. It adds to accumulating evidence (64,76) against the contention that blebbing is an unhealthy cellular process related to environmental abnormalities. It is possible that blebs may themselves be capable of initiating pseudopods (75). Exocytosis can be induced when the intracellular Ca^{2+} concentration is raised by the incorporation of a calcium ionophore into the cell membrane (77). Mg^{2+} cannot substitute for Ca^{2+} (78), and since ATP is required after Ca^{2+} influx (78), it is probable that the mechanism is more complex than simple charge neutralization or lipid segregation, which has been used to explain the role of Ca^{2+} in the fusion of negatively charged liposomes (79).

A hypothesis describing molecular events associated with exocytosis events by Lawson et.al. (62) suggest a triggered influx of Ca^{2+} leads to a multifocal lateral displacement of cytoplasm and

proteins in plasma and underlying granule membrane (possibly mediated by contractile elements, such as microfilaments) which allows the two membranes to fuse; the fused lipid bilayers, being depleted of protein and thus, perhaps, unstable, bulge from the cell to form a simple bleb, which subsequently vesiculates and pinches off. It seems likely that at least some of these postulated events occur in exocytosis. It has been shown that blebbing can be induced with various agents (81-88), including aldehydes (89), cytochalasins (86), local anesthetics (90), heat (90), and the peptide antibiotic EM 49 (91). It has also been reported to occur in cases of viral transformation (92). The extent to which cellular blebbing occurs in vivo is unknown.

In order to probe the architecture and the dynamics of the chlorosulfolipid membrane it is necessary that large quantities of pure sample be obtained. In the case of O. danica many attempts to isolate the plasma membrane have been unsuccessful. An alternative membrane preparation was sought. We have taken advantage of the observation that O. danica produces vesicles of plasma membrane which are extruded from the cell surface into the media (blebs) by exocytosis. Two simple, rapid procedures for isolating extruded plasma membrane vesicles from O. danica were developed. The vesicle preparations were analyzed for their lipid, protein and carbohydrate content in order to compare them to the previously characterized flagellar membrane (25,31). It was found that the vesicles of extruded O. danica membrane like the flagellar membrane, lack phospholipids and are constituted primarily of chlorosulfolipids. This suggests that the entire plasma membrane is made of these unusual

lipids. The membrane may be considered a model for acid membranes. Such membranes made exclusively of anionic lipids which is widespread in nature. Studies were conducted on the purified extracellular vesicles which suggest that this unique membrane is held together by unusual hydrogen bonds involving acidic protons.

EXPERIMENTAL PROCEDURE

Materials:

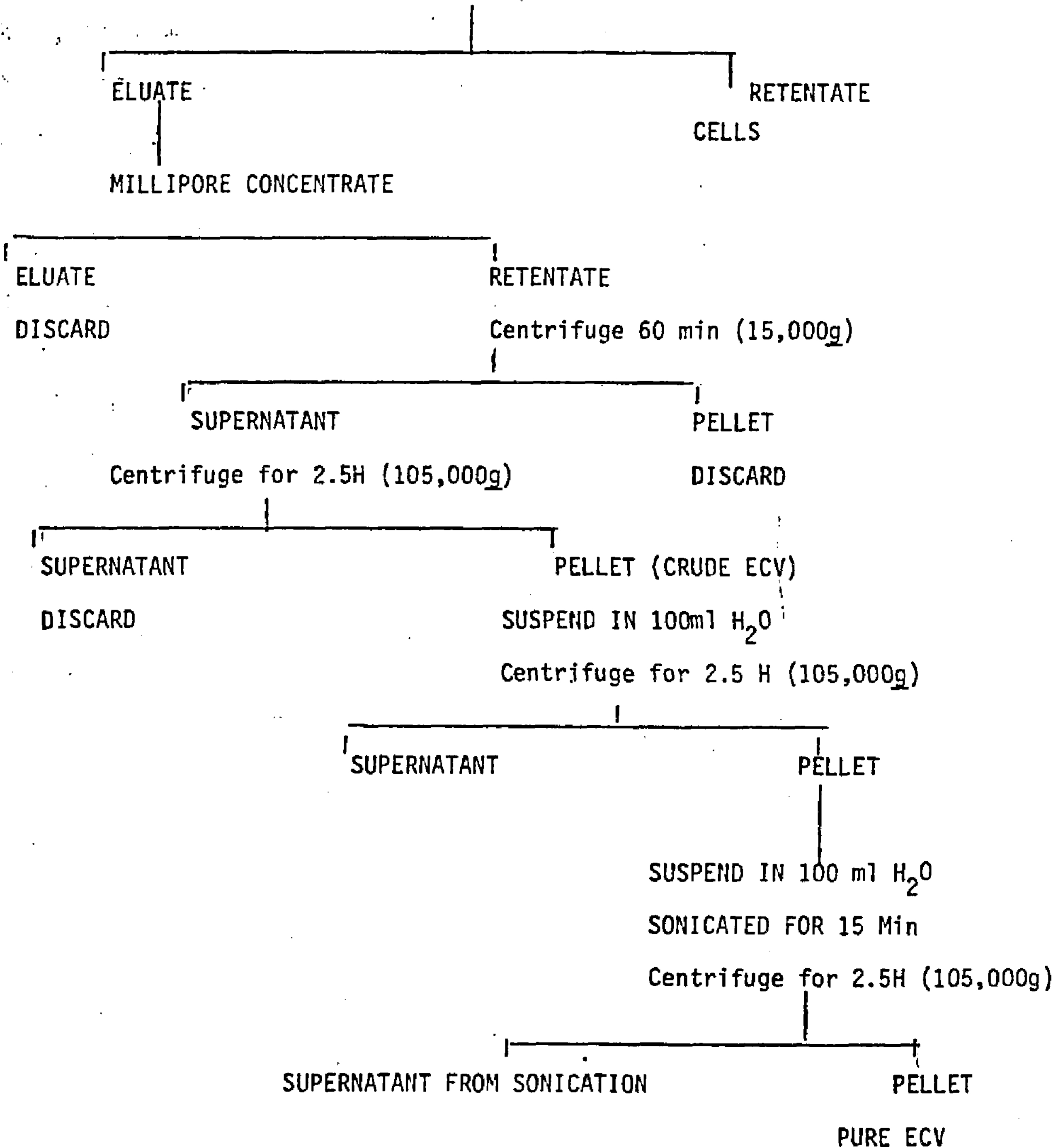
All reagents were analytical grade. Solvents were redistilled within a few days of use.

Cultures: *O. danica* was grown in the chemically defined medium of Aaronson and Baker (95) at 23° (pH 4.5) in ambient light. Cultures were harvested 5 days after inoculation.

Isolation of Extracellular Vesicles [ECV]: Two procedures were used to isolate the ECV. The first was based on differential centrifugation (Scheme I) after concentration of vesicular material in a Millipore Pellicon cassette system which provided a crude preparation of ECV's. A typical run was conducted as follows: the culture (15 liters) was first passed through the Millipore cassette system loaded with a (.45 micron, filter), (Millipore Corporation), at a flow rate of 125 ml/min. Whole cells were recovered from the retentate, the total volume of which was about 500 ml. The eluate (14.5 liters) was then immediately passed through the system at the slower flow rate of 35 ml/min using a finer filter (100,000 MW) and concentrated to a 500 ml-volume retentate. Conditions for the differential centrifugation of this ECV preparation are described in Scheme I. Sonication was conducted after resuspension of the pellet in 100 ml of H_2O with Fisher Model 300 set at 0.6 relative output (approx. 40 watts) using a microtip for 15 min. This procedure gave a pure preparation of sonicated ECV (Preparation I).

The second precedure used to isolate ECV was as follows: The cell

SCHEME I



culture was centrifuged for 20 min at 25,000g. The resulting supernatant was centrifuged at 113,000g for 1.5 hr. The supernatant was discarded, the pellets resuspended in fresh media, and the suspension centrifuged at 80,000g for 1 hr. The pellet was discarded. This step was repeated until a pellet was no longer obtained. The supernatant was then centrifuged at 113,000g for 1.5 hr to obtain a pellet of pure ECV (Preparation II).

Electron Microscopy: Preparations were fixed with 2% (w/v) glutaraldehyde in 0.2M cacodylate buffer, pH 7.0 at 4°C, and post-fixed for 1 hr in 1.0% (w/v) osmium tetroxide prepared using the same cacodylate buffer and temperature. Pellets were sequentially dehydrated with 50, 79, 95 (v/v) aqueous ethanol, and absolute ethanol, infiltrated over night and embedded in Spurr's resin. After sectioning with a Porter-Blum ultramicrotome (MT-5000), samples were supported on copper grids and post-stained with lead citrate (94) and uranyl acetate. Electron micrographs were obtained from a Philips 300 electron microscope.

Lipid Analyses: Lipids were extracted from the preparations with twenty volumes of redistilled chloroform: methanol (2:1, v/v) three times. The procedure was conducted under nitrogen as were subsequent procedures with lipids as deemed appropriate. BHT (0.01% w/v) (2,6-di-*tert*-butyl-4-methyl-phenol) was added to samples where storage was required. Lipid preparations were not taken to complete dryness except for aliquots used to obtain the dry weight. Weights of mg quantities were obtained on a Ventron Microbalance, Paramount, CA.

Fatty Acid Analyses: Gas liquid chromatography (GLC) was conducted on

a Perkin Elmer 881 Gas Chromatograph (flame ionization) using either an 8 foot column of SP2250 (3% w/v) on Supelcoport (80/100 mesh) or a six foot column of SP2330 (10% w/v) on Chromosorb WAW (100-200 mesh), the columns were obtained from Supelco, Bellefonte, PA. Fatty acid analyses were conducted on two fractions which yielded identical compositions. These were the fatty acid methyl esters and the free fatty acids. The latter were transmethylated with 2,2-dimethoxypropane in dry methanolic HCl (3N). The former was obtained directly from extraction and is presumably due to the extreme natural acidity of the membrane preparation during extraction with chloroform-methanol.

Thin Layer Chromatography TLC: TLC was conducted on Silica Gel 60 F-254 plates (0.25 mm) obtained from Brinkman Instruments, Westbury, N.Y. The solvent used for the separation of non-polar lipids was hexane: diethyl ether: methanol (60:40:2) (v/v/v). Polar lipids were separated on the same plates using the 2-dimensional solvent system of Rouser et.al.(95). [The first solvent (Rouser I) chloroform: methanol:ammonium hydroxide (65:35:5)(v/v/v), the second solvent (Rouser II) chloroform:acetone:methanol:acetic acid:H_2O (50:20:10:10:5) (v/v/v/v/v)

The Azure A Colorimetric Assay for Sulfolipid: The procedure of Kean (96) was used. Samples were pipetted into screw cap test tubes. To each tube was added 5.0 ml of chloroform:methanol, 1:1 (v/v), 5.0 ml of 0.05N H_2SO_4 and 1.0 ml of Azure A solution (40 mg in 5.0 ml of 0.05N H_2SO_4 diluted to 100 ml with water). The tubes were capped, vortexed for 30 sec and centrifuged (300*g* for 5 min). The absorbance (Gilford Instrument, Spectrophotometer-240) 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 Assays: Protein was assayed by the procedure of Lowry et al. (97) using bovine serum albumin (BSA) as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Buchler Electrophoresis system (Model 3-1751A) at pH 8.3 at 3 ma per gel using a modification (98) of the procedure of Shapiro et al. (99). The buffer (1 liter) contains 3 gm Tris(hydroxy methylaminomethane), (Sigma, Chemical Company, St. Louis, Mo.) 14.4 gm glycine, and 1 gm SDS. Protein samples (1-3 mg/ml) were dissolved in 0.0625M Tris, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue, and 5% (w/v) mercaptoethanol. The samples were applied to the gel after boiling for 5 minutes. Protein was stained using 1% (w/v) Coomassie blue solution in 10% (v/v) acetic acid and 30% (v/v) methanol. Gels were destained overnight in the same solution without dye.

Periodic acid Schiff Procedure: Gels were stained for carbohydrate using the periodic acid Schiff procedure. The SDS was removed by the following step; the solutions were stirred vigorously at room temperature for the followint stated times; no less than 50 ml per gel were used at each step (1) 25% (v/v) isopropyl alcohol, 10% acetic acid; overnight; (2) 10% isopropyl alcohol, 10% acetic acid; 6-9 hours; (3) 10% acetic acid; overnight; (4) 10% acetic acid; several hours. The fixed gels were then treated as the following: (1) 0.5% periodic

acid; 2 hours; (2) 0.5% sodium arsenite, 5% acetic acid; 30-60 min; (3) 0.2% sodium arsenite, 5% acetic acid; 60 min - repeated twice; (4) acetic acid; 10-20 min. The gels were then (5) transferred to tubes containing 10 ml of Schiff reagent and left overnight; (6) 0.1% sodium metabisulfite, 0.01 N HCl, for several hours, repeated until the rinse solution failed to turn pink upon addition of formaldehyde.

Anthrone: Total carbohydrate was routinely assayed by the anthrone method (100) using hexose as standard.

Methanolysis of the Carbohydrate: 2N Hydrochloric acid (5 ml) was added to the dry sample to be analyzed (80-90 mg) and the tube was tightly capped and heated in a boiling water bath for 2 hrs. The hydrolysate was neutralized by passage through a mixed-bed ion-exchange resin (Dowex 1 in the chloride form and Dowex 50 in the H^+ form) column (1 cm x 10 cm). Samples were evaporated to dryness *in vacuo*. Anhydrous methanolic HCl (1.5N) (Supelco) was added to each dried fraction, the samples are tightly sealed with teflon-lined screw caps and heated at 67°C for 11-24 hrs (32). A molar excess of silver carbonate (Pfaltz Bauer, Inc., Stanford, Conn.) was added and the solid precipitate was washed three times with approximately five volumes of methanol. The methanol washes were filtered through a Whatman 50 paper, and concentrated to dryness. Water (1 ml) and chloroform (1 ml) were added and the phases separated. The water phase was washed three times with 1 ml of chloroform. This procedure is necessary because the hydrolysis cleaves the sulfatides and the resulting diols contaminate the methyl glycoside preparation. The water phase contains the methyl glycosides and the chloroform wash contains the

diols (Scheme II) (32). The methyl glycosides of the ECV, together with prepared methyl glycoside standards were spotted on an analytical silica gel plate run in benzene:ethanol:water: ammonia (15N) (100:100:15:1) (v/v/v/v/) (32). The plate was visualized by spraying with 3% (w/v) H_2SO_4, 2.5% (w/v) $NaHSO_4$ followed by heating at 450°C.

Elemental Analysis: The elemental analyses were carried out by Schwartzkopf Microanalytical Laboratories (Woodside, N.Y.), using the following procedures: carbon, hydrogen: combustion at 900-1000°C under oxygen followed by gravimetric end analysis; nitrogen: Kjeldahl with colorimetric end analysis; Sulfate: precipitation as $BaSo_4$; Mg^{++}, Ca^{++}, Na^{+}: all determined by atomic absorption spectroscopy; chloride: colorimetric end analysis.

Albumin Treatment: BSA (fraction V) (Sigma)(0.1% w/v) was used to remove free fatty acids from ECV as described (104).

ECV (90 mg, dry wt.) was suspended in 6 ml deionized water, vortexed and divided equally. To one tube was added 5 µl BSA (0.1% w/v). Centrifuge for 2.5 hr (105,000g) and the supernatant was removed. Fresh deionized water (3 ml) containing 5 µl of BSA solution was added to the ECV preparation that had been exposed to the BSA and the centrifugations repeated. The supernatants were combined. Supernatant (1 ml) was evaporated to dryness under a stream of nitrogen. The residue was extracted with a (1 ml) of hexane. (Identical results were obtained using chloroform, methanol and mixtures as extracting solvents).

Approximately 60 µl of the hexane was spotted on the TLC plate together with a portion of the moist pellet of BSA-extracted ECV.

SCHEME II

Separation of Methyl Esters of Fatty Acids, Chlorodiols and Methyl Glycosides formed from Methanolysis in Anhydrous HCL of the ECV

the ECV (80 mgms)

a. 1.5N anhydrous methanolic HCl at 67°C for 16-24 hrs.
b. Neutralize with silver carbonate.
c. Wash three times with wet methanol and concentrate the combined washes to 1 ml.
d. Extract three times with equal volumes of hexane.

Upper Phase (combined hexane extract)

Predominantly methyl esters of fatty acids plus perchlorinated diols.

Lower Phase (wet methanol)

a. Concentrate to dryness and add 1 ml water.
b. Extract the water three times with equal volumes of chloroform.

Upper Phase (water)
Methyl glycosides

Lower Phase (chloroform)
Chlorodiols

In addition the appropriate controls of unextracted supernatant and pellet were chromatographed.

The samples of supernatant and pellet (both BSA-extracted and control) were analysed for total lipid (gravimetric), carbohydrate (anthrone) and protein (Lowry).

pH Fluorescent Indicator Studies: Two types of alkylated coumarin dyes were used as pH fluorescent indicators, hydroxycoumarin (4-undecyl-p-hydroxycoumarin) and aminocoumarin (4-heptadecyl-p-aminocoumarin). Aklylated coumarin pH indicators were first used by Fromherz (39) to measure the pH at polyanionic lipoid surfaces.

The two types can be used as acid-base indicators. In the case of hydroxycoumarin only the basic form (resulting from the dissociation of hydroxyl group) fluoresces at 450 nm when excited at 366 nm. At a given concentration of the indicator the amount of the fluorescence is proportional to the degree of dissociation of the hydroxyl. Therefore the pK can be obtained from fluorescence and pH measurements.

In the case of aminocoumarin only the acid (protonated form) fluoresces. This indicator can be used to examine the lower pH (0-4) while hydroxycoumarin examines the upper pH range (6-12).

In order to explain the unusual pH properties of anionic lipid surfaces and the use of these indicators in studying their properties, Fromherz has developed the appropriate theoretical basis for these measurements (39).

The indicators were provided for our use as a gift from Dr. P. Fromherz. [Max-Planck-Institut fur Biophysikalische, Gottingen, Germany]. Amino coumarin solution A was prepared by dissolving 1 mg of the amino

coumarin in 4 ml of chloroform:methanol (1:3). Each solvent was Spectro Grade (Fisher Scientific Company, N.J.). Solution B (hydroxycoumarin) was prepared by dissolving 6 mg of the hydroxycoumarin in 6 ml of methanol. ECV (200-500 μl of a solution containing 35 mg of dry weight per ml) was diluted 6 times with distilled, deionized water. Measurements were made on a Perkin Elmer Fluorescent Spectrophotometer (MPF-2A). Excitation was set at 366 nm; filter #43 was used; slit settings were varied with solutions and were maximized to reduce background. The slit was varied from 6-10 for both the emission and the excitation slits. Emission scanning was between 400-600 nm. The emission peak for both indicators was at 450 nm. To determine the pK of the hydroxycoumarin indicator at the vesicle surface, a 3 ml dispersion of the diluted vesicles was adjusted to pH 11.7. After scanning the vesicle preparation in the spectrometer to determine a minimal blank, 5 μl of the stock solution A was added to the sample and scanned. The pH was adjusted to 11.9. Upon this adjustment the emission spectrum remained unchanged: the peak height was at a plateau The indicator was considered completely dissociated. An increment of HCl was added to conduct the titration. At discrete points along the titration curve, the fluorescent peak was scanned. At the pH at which there was no change in fluorescence from the previous pH, the titration was considered complete. The fluorescence peak heights were measured, corrected for volume changes, and the percentage dissociation determined. The latter was calculated by dividing each peak height by the peak height of total dissociation (e.g. pH 11.7 plateau). These percentages were then plotted and 50% dissociation

was considered the pK. The same procedure was used for the hydroxycoumarin indicator. A 3 ml dispersion of vesicles (pH 4.1) was adjusted to pH 6.6 which was the plateau region for complete dissociation. This was considered blank and the indicator (20 µl of stock solution B) is added. The pH was adjusted with HCl and the solution was scanned at various pH's to pH 0.8. The points were calculated and plotted as before, and the pK was determined (50% dissociation).

Results

<u>Preparation and Composition of Extracellular Vesicles(ECV)</u>

Two procedures were used to obtain preparations of extracellular vesicles (ECV). The concentration of a cell-free supernatant with the Millipore filtration system followed by sonication of the vesicles may be considered a vigorous isolation procedure (Preparation I). In contrast an isolation based on differential centrifugation is considered a gentle procedure (Preparation II). The electron micrographic and analytical studies conducted on these preparations demonstrated a significant difference in the quality of each ECV preparation.

Preparation I was examined in the electron microscope (Fig. 5,6). The vesicles are trilamellar and of relatively uniform size. The electron micrograph demonstrates the purity of the membrane preparation. The isolation was followed by electron microscopy. This assay showed the necessity of each step in obtaining a pure membrane preparation.

Preparation II was a less pure preparation. Electron micrographs showed it contained occasional rods, presumably tubular mastigonemes. Furthermore the vesicles were widely variant in size.

Compositional studies were conducted on the two preparations with respect to the protein, lipid and carbohydrate. At least five determinations were made on each preparation. The results showed (Table I) a narrow range of variation in the composition regardless of the method of preparation of the membrane. It appears, then, that the ECV have a consistent composition which is significantly different than that of the flagellar membrane (Table I). The latter was studied by

Figure 5. Electron Micrograph of the ECV Preparation I. Low Magnification, (X 20,000).

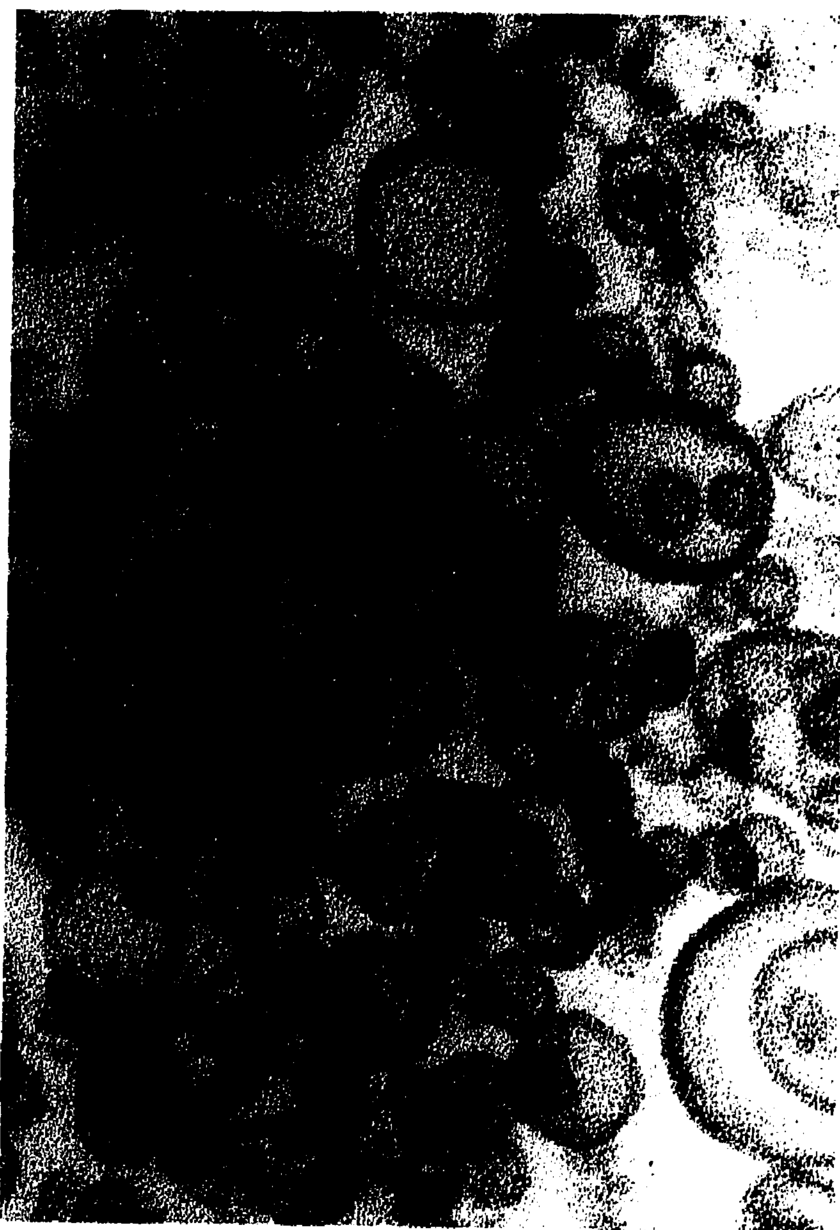


Figure 6. Electron Micrograph of the ECV Preparation I. High Magnification (X 90,000).

TABLE I

MEMBRANE COMPOSITION

Percent Total Weight

	Flagellar Membrane	Extracellular Vesicles (ECV)
PROTEIN	28[1]	14-18[1]
LIPID	10[2]	26-28[2]
CARBOHYDRATE	less than 2	35-40[3]
UNKNOWN	61[4]	19[4]

The flagellar membrane results are taken from Stern et al. (36)

1. Determined by Lowry and by Nitrogen analysis.
2. Determined gravimetrically after extraction with (chloroform:methanol 2:1 v/v)
3. Determined by anthrone which does not detect amino sugars. TLC of the component sugar residues (Fig. 9,10) showed the amino sugar component to be approximately 10% of the carbohydrate. Amino sugar is included as such in the total percent indicated.
4. The unknown represents the unaccounted substances.

Chen et.al. (31,25) and by Stern et.al. (36).

Examination of the lipids of the ECV preparations showed that the lipid composition was essentially that of the flagellar membrane. There were no detectable phospholipids and only trace quantities of polar lipids other than the chlorosulfolipids (Fig. 7). There was a slightly larger amount of unknown polar lipids (8.8%). As was the case for the flagellar membrane the chlorosulfolipids represented about 70% of the total lipids. The non-polar lipid pool was approximately the same as that of the flagellar membrane (22.4%), Fig. (8), Table II the proportion of free fatty acids was greater.

An important distinction between the two ECV preparations is the extent of hydrolysis of chlorosulfolipids which occurs during the isolation of (preparation I) especially during filtration with the Millipore Pellicon cassette system and during the subsequent sonication. As much as 55% of the total sulfolipid in the membrane is hydrolyzed. It was for this reason that Preparation II (a gentler isolation procedure) was undertaken. The latter yielded essentially no hydrolysis products (diols).

Free fatty acids had been reported in the flagellar membrane of O. danica (25). These free fatty acids appear not to be due to lipase activity or artifacts (25). The free fatty acids were scraped from a TLC plate, extracted from the silica gel (repeated twice) under nitrogen with chloroform-methanol 2:1(v/v) and analyzed by GLC as the methyl esters. The results are shown in Table III. It should be noted that the methyl esters showed a composition identical to that of the free fatty acid fraction. This was also the case for the

Figure 7.

Fig. 7. Thin Layer Chromatogram (TLC) of the polar lipids of the extracellular vesicles (ECV) from O. danica. The lipid extract was spotted on the lower right hand corner. Solvent system 1, chloroform:methanol:% (w/v) aqueous ammonia (65:35:5) (v/v/v).

Solvent system 2, chloroform:acetone:methanol:acetic acid:water (5:2:1:1:5) (v/v/v/v). The series of spots in the center of the plate are the chlorosulfolipids. The heaviest is the hexachlorocompound. The arrow indicate the free fatty acids. All of the typical phospholipids are absent from the plate.

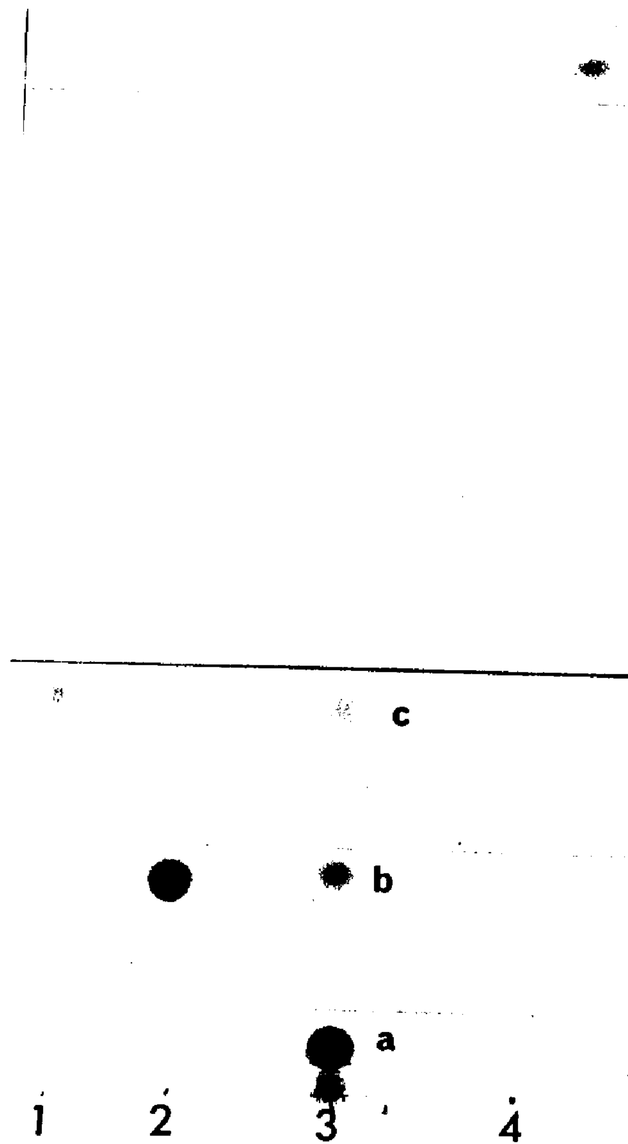


Figure 8.

Fig. 8. Thin layer chromatogram (TLC) of the non polar lipids of Preparation I of the extracellular vesicles (ECV) from O. danica visualized by charring with 25% (w/v) $NaHSO_4$ containing 3% (v/v) H_2SO_4. The plate was developed in ether:hexane (3:7) (v/v).

1. oleic acid standard
2. cholesterol standard
3. lipid extract of the ECV
4. methyl oleate standard

(a) Chlorodiol mixture. These are the result of hydrolysis of sulfolipid during preparation of the sample.

(b) a mixture of the six sterols (115).

(c) free fatty acids mixture (Table 3).

TABLE II

LIPID COMPOSITION OF THE ECV

Polar Lipids		
Sulfolipid	69.1	
Polar Unknown Lipids	8.8	
Total Polar Lipids		77.9
Non-Polar Lipids		
Free Fatty Acids	13.9	
Sterols	7.0	
Non-polar Unknown	1.2	
Total Non-polar Lipids		22.1

TABLE III

FREE FATTY ACIDS

Chain Length	Double Bonds 0	1	2	3	4
14	13.9				
16	13.7	2.8			
18	24.9	7.2	4.5		
20	2.4	4.9	1.1		
22	3.7	2.9			

The free fatty acid fraction was converted to the methyl esters by the use of 2,2-dimethoxypropane (DMP) to drive the transesterification reaction to completion and eliminate the need for elevated temperatures (102). Values are expressed as % composition based on a gas chromatogram of the methyl esters.

flagellar membrane. Previous experiments (103) had demonstrated that extraction using ethanol produced ethyl esters. These results imply that the isolated fatty acid methyl esters are esterified by the extracting solvent. This is supportive evidence that the fatty acids are unesterified in the membrane. It also suggests that the membrane is unusually acidic. It should be noted that the total saturated free fatty acids in the ECV was 58.5%, whereas that for the flagellar membrane (25) was only 38.4%. The high level of saturation in the free fatty acids is unusual for O. danica.

The Carbohydrate: Hydrolysis of lipid extracted ECV (preparation I) followed by methanolysis permitted a sugar analysis of the membrane. The TLC Fig. 9, Fig. 10 of the methyl glycosides shows that the hydrolysis was complete and the major neutral sugar in the ECV s mannose. This is different from that of the flagellar membrane which is rhamnose (32). Furthermore it is clear from Table I and Fig. 10 that the carbohydrate dominates the ECV whereas in the flagellar membrane it is minor. It is probable that the flagella has a single oligo-saccharide carbohydrate composition is quantitatively reduced to simple whole numbers of relative amounts of its sugar constituents. In contrast the ECV is likely to have many types. It should be noted that further investigation of the composition of the ECV carbohydrate would be necessary to establish the quantitative amounts of each sugar and to obtain the amino sugar content

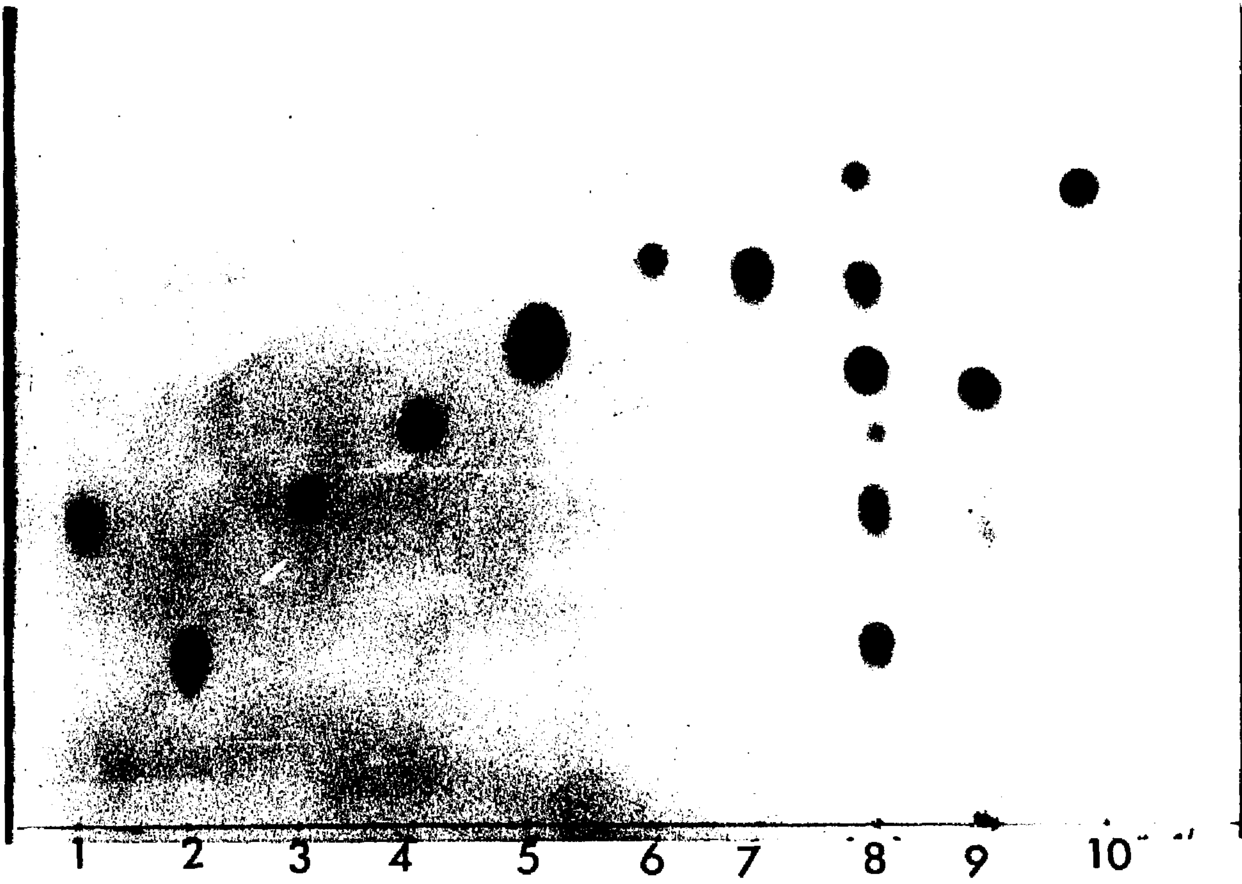


Figure 9.

Fig. 9. Thin layer chromatogram of methyl glycosides of the ECV and of standards.

Lane 1: methyl-N-acetyl-D-glucosaminide

Lane 2: glucose

Lane 3: methyl-D-galactoside

Lane 4: methyl-D-glucoside

Lane 5: methyl-D-mannoside

Lane 6: methyl-D-xyloside

Lane 7: methyl-L-fucoside

Lane 8: combination of all standards

Lane 9: the methyl glycoside mixture from the ECV

Lane 10: methyl-L-rhamnoside

The plate was developed by one ascending run in benzene: ethanol:water:15 Molar ammonia (100:100:15:1) (v/v/v/v).

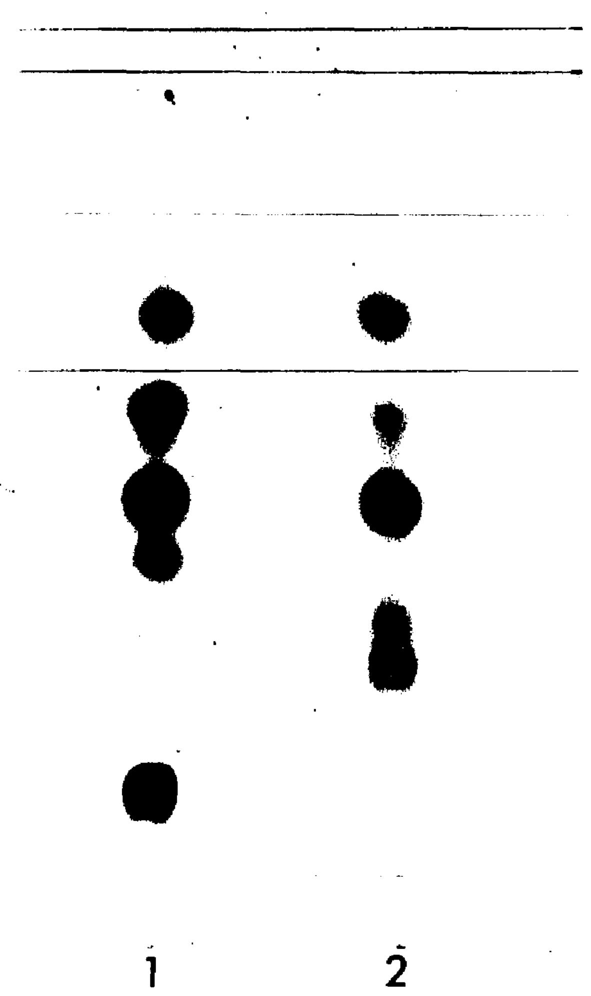


Figure 10.

Fig. 10. Thin layer chromatogram of the methyl glycosides of the ECV.
Lane 1: combination of all standards as in Fig. 9
Lane 2: the methyl glycoside mixture from the ECV.
The plate was developed by one ascending run in benzene: ethanol:water:15 Molar ammonia (100:100:15:1) (v/v/v/v).

of the ECV.

It should be noted that the amino sugar analysis of the glucosamine flagellar membrane contained only one amino sugar, N-acetyl glucosamine and that it represented 10% of the total sugar of the flagellar membrane (32). In addition analysis of the chrysomonad, Ochromonas malhamensis, a closely related organism showed (104) that it had a chitin (N-acetyl glucosamine) pellicle protecting it under certain growth conditions.

The Protein:

The electrophoretic analysis of the ECV proteins stained with Coomassie blue are shown in Fig. 11. Some protein (about 15%) remained on the top of the gel. Any protein having a molecular weight substantially greater than 120,000D would remain at the top and was not further investigated.

The ECV contain four major proteins having apparent molecular weights of 97,000, 85,000, 47,000 and 19,000D. In addition one minor protein band of apparent molecular weight of 110,000D. Each of these four bands were positively stained with the periodic acid-Schiff reagent which showed that they were glycoproteins. This observation gives less confidence to the"apparent" molecular weights since it is well known that glycoproteins behave erratically in SDS gel electrophoresis with respect to the determination of molecular weight.

It should be noted that the supernatant that was equilibrated with the so icated ECV ["supernatant from sonication" Scheme I] gave an identical electrophoretic pattern (Fig. 11). This indicates that the protein of the ECV is released to the supernatant by sonication.

Figure 11.

Figure 11

1. Supernatant after sonication of the ECV followed by centrifugation at 105,000 g for 2.5 hr.
2. The ECV (isolated at 105,000 g for 2-5 hr)
3. Standards: (Top of gel) phosphorylase b (94,000); bovine serum albumin (68,000); oval albumin (45,000); chymotryspin (25,000); tracking dye.

The dry samples were solubilized in 2% (w/v) SDS, 1% (v/v) mercaptoethanol, 0.625M Tris buffer (pH 6.8) and electrophoresed on SDS 10% (w/v) arcylamide gels for 4.5 hrs. The standards were dissolved in 1% (w/v) SDS as a kit from Sigma Chemical Co.

Elemental Analysis of the Extracellular Vesicles (ECV)

A single determination of the elemental composition of the membrane (Preparation I) was conducted. The analyses were conducted as follows: (Table IV).

Metal ions were determined by atomic absorption. Nitrogen was determined by Kjeldahl. The total nitrogen less the amino sugar nitrogen multiplied by 6.38 was considered to be the total protein. The total was 14% which was not very different from the 18% determined by the method of Lowry et al. (97) using bovine serum albumin as a standard. The sulfolipid content is based on the total sulfur and on the chloride analysis as well. The sulfur analysis was based on oxidation of the sulfur to sulfate and its precipitation as $BaSO_4$.

The analysis allows us to consider the total quantitation of sterols, fatty acids and the small amount of uncharacterized lipids to obtain an overview of the membrane. Thus the protein to lipid ratio and the carbohydrate composition taken together allows us to estimate that some 20% of the substance of the membrane is not accounted for. The elemental composition of this material suggests it is low in oxygen and contains no nitrogen, phosphorous, or sulfur. The analysis also shows that all of the nitrogen is accounted for in the protein and that no metals and therefore no counterions are present to neutralize the secondary sulfates in the hydrophobic domain of the bilayer. In the study on the flagellar membrane (36) the amino acid composition showed that the basic amino acids of the protein could not serve as a counterion. Although no amino acid was conducted on the ECV, it seems unlikely that the protein could serve as a counterion to the secondary sulfates in the hydrophobic domain of the bilayer.

TABLE IV

ELEMENTAL ANALYSIS

	Total % Weight	C	H	O	N	S	Cl^-
Protein[1]	14-18%	8.34	1.32	5.53	2.18	<0.01	
Lipids							
Sulfolipids[2]	18.4	8.15	1.2	4.0		0.98	0.55
Sterols[3]	2.3	1.9	0.2	.07			
Fatty Acids[3]	3.65	2.77	0.4	0.4			
Unknown Polar[3]	1.8	1.2	.20	0.4			
Unknown Non-polar[3]	0.4	.34	.12				
	26.5						
Sugar							
I. Neutral Sugar[4]	33	14.5	2.31	16.17			
II. Amino Sugar[5]	3.5	1.4	.23	1.5	0.23		
Unknown (Calculated by difference)[6]	19	16.81	2.09	3.67			
Found		54.61	8.07	31.74	2.41	.98	0.55

Table IV (Continued)

1. Protein based on the Lowry is approximately 18% (± 1%). Nitrogen analysis (2.41%) less the estimated amino sugar nitrogen content (0.23%) is 2.18%. Use of the multiplier, 6.38, indicates a protein content of 14%.
2. The value (18%) is based on weight of the sulfolipids as a fraction of the total lipids (Both Preparation I, II). Less sulfolipid (10%) was found by both the Kean assay and the sulfur analysis (0.98%). These were found to produce hydrolysis products. Hydrolysis should decrease the yield. The higher value is thus considered more reliable.
3. Value established by proportional weight of the total lipids. Average molecular weights for molar calculations are 407 (sterols), 276 (fatty acids), 728 (based on digalactosyl diglyceride as an unknown polar lipid), and hydrocarbon C:H ratio (non-polar lipid).
4. Estimated from anthrone using hexose standard. For the flagellar membrane (1.3% total CHO) the anthrone assay produced a charred material that made its use unacceptable. In contrast the ECV membrane preparation gave a persistently high anthrone analysis.
5. Methyl glycosides were examined on TLC after digestion of the sample with 1.5N methanolic (anhydrous) HCl for 11-24 hrs. at 67°. The chromatograms showd that approximately 10% (±5%) of the total sugars of the oligosaccharide was amino sugar.
6. The elemental composition of the entire membrane for C, H, O (by difference), Cl, S, P and ash (none detectable), less the elements of protein, lipid and carbohydrate that are known to be present indicates that substance(s) remains unaccounted for. The elemental composition of the unknown material indicates that it contains only C, H and O, with C and H predominant. This unknown represents about 1/5 of the total sample.

Studies on Membrane Stability:

It is apparent from a comparison of the lipid composition of the two procedures used for the preparation of the ECV that hydrolysis of chlorosulfolipids occurs during the use of Millipore filtration followed by sonication. It is assumed that the hydrolysis of sulfolipids will effect the stability of this unique membrane because these lipids are the principal polar lipids of the membrane. The ECV preparation contains an unusually large amount of carbohydrate. One must consider that the analysis includes both the hydrophobic domain and the hydrophilic domain of the bilayer. One may not include the carbohydrate which is expected to be on the aqueous surface as contributing to the stability of the bilayer. That hydrolysis of sulfatide did not destroy the bilayer is demonstrated by the high quality of the bilayer seen in electron micrographic studies on Preparation I in which the sulfatides had been found to be extensively hydrolysed.

The evidence suggests that under such circumstances (extensive sulfolipid hydrolysis in intact membrane preparations) the membrane is stabilized by the free fatty acids together with the diol (derived from hydrolysis), the sterol, and the residual sulfatide.

Hydrolysis of the sulfatide in intact membrane preparations implies an unusual acidity of the membrane since aliphatic sulfates are stable under neutral and basic conditions but are readily hydrolyzed in acid. Such acidity is also consistent with the formation of fatty acid methyl estees during lipid extraction.

That unusual acidity is associated with a membrane made of anionic-

headgroup lipids and acidic proteins is to be expected from the electrostatics of the membrane as predicted by the Gouy-Chapman theory and was demonstrated by the pH indicator studies. Nonetheless, the acidity implied by the formation of fatty acid methyl esters and by hydrolysis of the sulfatides in intact membrane suggests that the protons that generate this acidity are not simply due to the electrostatic concentration of hydronium ions at the surface but are somehow entrapped within the membrane.

In order to establish whether or not protons are actually trapped at the surface (or within) the membrane the effect of sonication on the membrane at several pH's was examined. In addition (and as a control for the sonication study) it was necessary to examine simple resuspension of the membrane in deionized water. In these studies the release of sulfolipid, protein, and protons was assayed after resuspension and after the combination of resuspension with sonication.

Table V compares the protein and sulfolipid composition of the ECV under the specified conditions. From this table we can conclude the following:

1. Ten to twelve percent of the membrane is solubilized on resuspension in deionized water and this amount is more than doubled if the pH is adjusted to 8.4.
2. Sonication triples the extent of solubilization of the membrane in each of the two suspensions.
3. The fact that the protein:sulfolipid weight ratio (P:S-L) remains constant during these manipulations indicates that

solubilization is not due to selective solubilization of membrane components (sulfolipid or protein) but rather to the solubilization of the entire membrane. (The decrease in P:S-L ratio recovered in the pellet is presumably due to an artifact in the pellet resuspension procedure.) Equivalent samples had been examined in the electron microscope and shown to be pure membrane vesicles with very little non-membrane material present. It should be noted that electrophoresis of the supernatant after sonication yielded a protein pattern that was virtually identical to that of the membrane (ECV). This is consistent with the conversion of vesicles to small, seemingly soluble material by sonication.

Suspension in the high pH medium is shown to reduce the amount of pellet altogether presumably by dissolution of the components. Alternatively suspension in high pH provokes the formation of smaller vesicles or aggregates that are not pelleted by the same centrifugal conditions.

Several interesting conclusions about the stability of the membrane may be drawn from the data shown in the Table. These conclusions have important implications to the handling of the membrane in the laboratory. They also provide some insight into the biochemical and biological features of the membrane, the latter will be considered in the discussion.

In a separate series of experiments designed to establish the release of protons to the medium by resuspending the ECV in deionized

water, it was found that resuspension of the membrane results in a decrease of the pH. The molar quantity of protons released is approximately that of the sulfolipid solubilized. In a typical experiment a pellet of ECV containing 0.45 μmoles (282 μg) sulfolipid released 0.11 μmoles (69 μg) of sulfolipid into the supernatant upon resuspension in deionized water (pH 6.9) and simultaneously decreased the pH to 5.9. The latter implies a release of 0.1 μmoles of protons to the aqueous phase from the membrane. It thus appears that the solubilization of sulfatide is accompanied by release of protons from the membrane on a 1:1 basis. These results support the notion that the membrane harbors protons and that it releases them in a molar ratio with chlorosulfolipid from the bilayer. Sonicated membrane resulted in a greater release of sulfatide into the supernatant accompanied by the release of fewer protons. Presumably this is due to the formation during sonication of lower molecular weight aggregates which do not collect on centrifugation under the same gravity conditions. These aggregates appear to retain the trapped protons.

TABLE V

ECV PREPARATION II	pH Before	pH After	Total			Supernatant					Pellet by Diff.				
			P-mg	S-L mg	ratio P/SL	P	Recovery	S-L	Recovery	ratio P/SL	P	Recovery	S-L	Recovery	P:S-L ratio
											4	3	4	3	
Suspended in deionized H_2O centrifuge 2.5 hr.	6.9	4.1	1.7±.04	.34±.02	5:1	.16±.03	10%	.04±.02	12%	4:1	90%	42%	88%	47%	5:1
Suspended in deionized H_2O-centrifuge 2.5 hr.	8.4[1]	8.4	1.6±.04	.35±02	5:1	.37±.03	33%	.09±.09	26%	4:1	77%	41%	74%	40%	5:1
Suspended in deionized H_2O and sonicated centrifuge 2.5 hr	6.9	4.1	1.7±.04	.37±.02	5:1	.57±.03	34%	.11±.03	30%	5:1	66%	30%	70%	40%	3.4:1
Suspended in deionized H_2O and sonicated centrifuge 2.5 hr.	8.2[2]	7.8	1.7±.04	.4±.02	4:1	1.1±.02	64%	.25±.03	62%	4:1	36%	21%	38%	28%	3:1

Table V. Comparison of the release of protein and sulfolipid from the ECV Preparation II during resuspension in deionized water and at pH 8.4, and in each after sonication at 40 watts for 15 min.

P = protein

S-L = chlorosulfolipid

1 add 10 μl of 1N NaOH

2 add 10 μl of 1N NaOH

3 Attemps to assay the protein and the sulfolipid in the pellet were frustrated by an inability to resuspend the pellet homogeneously for the analyses. Thus only 50% of the pellet protein and sulfolipid could be found on repeated analyses. The fact that the ratios of protein to sulfolipid were constant in both the supernatant and pellet assays showed that the components were not degraded.

4 By differences

Studies of Free Fatty Acid Removal from ECV by Bovine Serum Albumin (BSA):

It is well known that BSA is the intravascular transport protein for free fatty acids (105), the specificity of binding of fatty acids to albumin has been studied extensively. The subject has been reviewed by Tanford (106).

The fact that O. danica contains a significant amount of free fatty acids in its membranes (flagellar membrane and ECV) suggests that BSA may be used to remove some of these free fatty acids from membrane preparations without disturbing the integrity of the membrane.

Samples of ECV were examined before and after treatment with BSA (Table VI). As was expected this treatment had a noticeable impact on the membrane's composition. Fig. 12 shows electron micrographs of the ECV after BSA treatment. It may be seen that multilamellar myelin-like membranes are formed by the removal of free fatty acids.

In earily experiment it has been found that excess albumin (20μl of BSA) caused the formation of a copious white precipitate which appeared in the electron microscope to be denatured albumin in that uniform particles about 60 nm in diameter were the dominant material in the precipitate.

TLC of both the supernatant and the pellet showed that most of the free fatty acids were removed together with a small portion of the sterols.

TABLE VI

BSA TREATMENT:

	Before BSA Treatment	AFTER BSA Treatment
Protein	16%	18%
Carbohydrate	35%	40%
Lipid	27%	22%

BSA used to remove free fatty acids from ECV

Figure 12. Electron micrograph of the ECV after treatment with BSA. The swirls (myelins) are a consequence of the treatment with the BSA which removes most of the free fatty acids from the bilayer. X 42,000.

pH Fluorescent Indicator Studies:

Two alkylated pH indicators were used hydroxycoumarin and amino coumarin (39). The fluorescence of each of these indicators are completely quenched at 450 nm by protonation. It is therefore possible to measure the extent of dissociation of the indicators by fluorescence. The pH at 50% dissociation is equal to the pK of the indicator. These indicators have previously been used (39) to examine the pH at membrane surfaces (within 1.0 nm). The pH in this region of polyanionic surfaces is known to be different than that of the bulk phase, as discussed by McLaughlin et al. (48). In lipid monolayers the indicators have been shown to be oriented with the alkyl chain buried in the bilayer with the coumarin head in or just above the headgroup region (39). The headgroup region is located just below the Stern layer (39). The indicator may thus be used to monitor the electrostatic potential at the surface of a negatively (or positively) charged membrane. Fromherz (39) has integrated the use of these indicators into current electrostatic theory. All of the experiments discussed are at negatively charged surfaces.

Previously Fernandez et al. (39) had shown that the shift in pK of the indicator when it is at a surface as compared to the same indicator in bulk solution is due to two properties of the surface: 1. the electrostatic potential (related to charge density at the surface of the membrane) and 2. a change in the polarity of the surface.

In order to sort out how each of these two properties affects the pK shifts of the indicators Fromherz used Triton X-100

micelles as a model for a "non-polar" or neutral surface. We have repeated these experiments with the same detergent and obtained results reported by Fromherz (39). Thus the shifts of pK observed in Triton X-100 are used to determine the "polarity component" of the total pK shift of the indicator. Fernandez has demonstrated that this polarity component is independent of whether the surface is uncharged due to the existence of zwitterionic charged (overall neutral) headgroups such as PC or uncharged, but water soluble groups (glycolipids, Triton X-100) at the surface (39).

In electrically charged systems, such as SDS micelles, (overall negative) there is a shift of the pk of the indicator to higher pH (39). Thus the SDS micelles exhibit both a polarity and an electrostatic contribution to the pK shifts.

A reasonable question is how can these two effects be sorted out by the measurement of the pK's of the indicators. Aminocoumarin (pH range 1-4) is positively charged (protonated) below its pK and uncharged above its pK. Hydroxycoumarin (pH range 6-12) is uncharged (protonated) below its pK negatively and charged above its pK. As the polarity component is reduced at a membrane surface the uncharged form of the indicator binds preferentially. This results in a downward shift for aminocoumarin and an upfield shift for hydroxycoumarin. In contrast, the electrostatic effect shifts both indicators in the same direction and to the same extent from the "non-polar" position.

Fromherz (39) showed that both indicators exhibited identical pK shifts from the Triton X-100 position in systems that retained the same surface charge density in these widely variant pH's. The shifts apply

to any uncharged or zwitterionic lipid system.

For aminocoumarin which has a pK in water (no alkyl group) of 2.35 there is a downward shift of 1.25 for the alkylated species bound to Triton X-100 micelles. This shift of 1.10 pH units is due to the relative stabilization of the alkylated indicator in the uncharged form (deprotonated) to the non-polar surface. A symmetrical result is obtained for the hydroxycoumarin indicator. In this case the non-alkylated coumarin in water exhibits a pK of 7.75. The uncharged form for this indicator is the protonated form which, when the indicator is alkylated, is stabilized in the non-polar Triton X-100 micelles. Under these conditions the pK of the indicator is 8.85. Thus alkylated hydroxycoumarin due to its protonated (uncharged) form exhibits an upward shift of 1.10 pH units in the presence of Triton X-100 whereas alkylated aminocoumarin due to its deprotonated (uncharged) form exhibits a downward shift of the same 1.10 pH units in the presence Triton X-100. Since the two indicators have this shift in opposite directions the "non-polar binding effect" of the alkylated indicators can be separated from the "electrostatic binding effect". Since the the latter always shifts the pK of both indicators in the same direction (upward for negative surfaces and downward for positive surfaces).

It has been shown (39) that the apparent shifts of pK at a charged surface are in fact not true shifts in pK, but rather different of pH as compared to the bulk phase. Thus, the apparent shifts in the pK are numerically equal to the pH difference between the bulk phase and the membrane surface.

To measure the pH at the surface (as compared to that at the bulk phase) one subtracts the pK of the indicator at a non-polar surface (Triton X-100 micelles) from the measured pK at the surface of interest and reports this difference as the pH differential at the surface in question. For example in SDS micelles alkylated hydroxycoumarin shows a pK of 11.15 which means that the surface pH is 2.3 units lower (11.15 less 8.85). Likewise alkylated aminocoumarin exhibits is 2.3 units (3.55 less 1.25) lower than the bulk phase. The pK of the indicator is thus 8.85 for hydroxycourmarin thus SDS micelles have a pH differential of 2.3 pH units (lower at the surface than in the bulk phase) through the entire range from pH 3 to pH 11. This means that the surface charge density does not change in this pH range.

The introduction ofcarboxylates into an alkyl sulfate surface introduces a further complication. At low pH the carboxylates are protonated wheras, at high pH they are deprotonated (negatively charged). Thus, the charge density at the high pH is greater in a mixed alkyl sulfate-carboxylate system than it is at the low pH. It may been seen in Figure 13, that the charge density at the surface is different at pH 9-11 (hydroxycoumarin) than it is at pH 1-3 (aminocourmarin), for both the ECV and the model membrane system which contains free fatty acid (oleic acid).

Table 7 shows the results in tabular form for the ECV. Preparation I and Preparation II yielded identical curves.

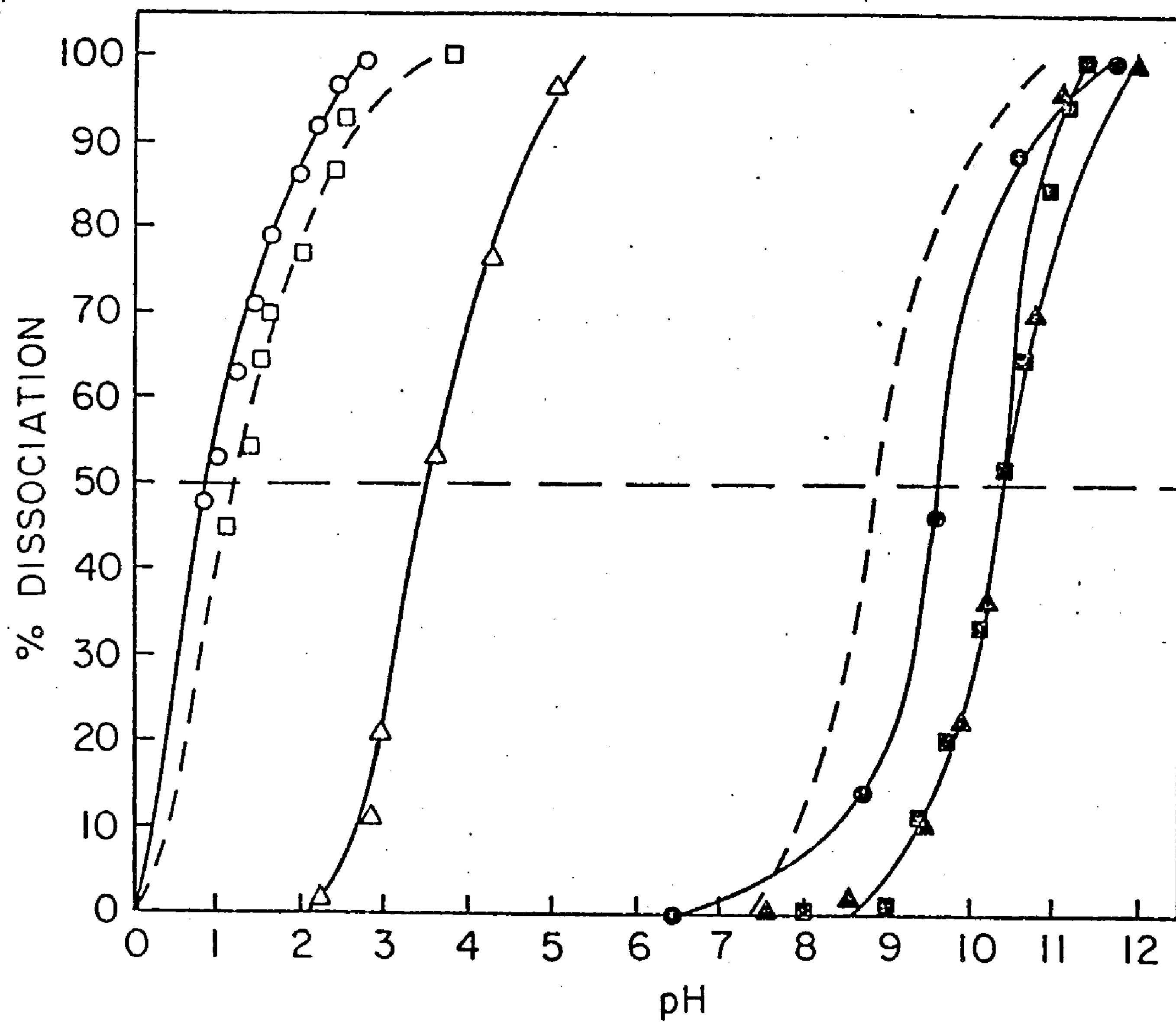
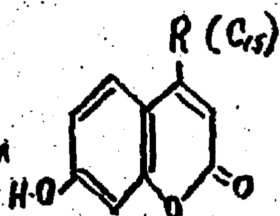
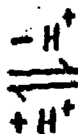
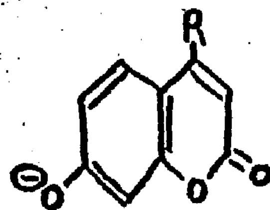
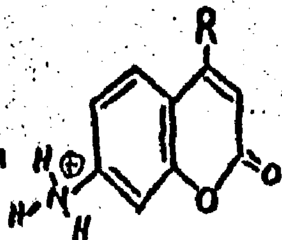
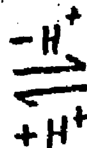
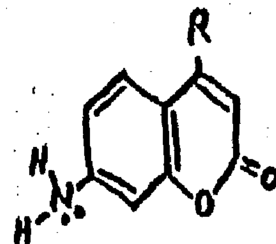


Fig. 13

Figure 13. Percentage dissociation of pH-indicator vs. bulk pH. The graph compares the alkylated indicator pair hydroxycoumarin (closed markers) and aminocoumarin (open markers) hydrophobically bound to surfaces. The broken line refer to the respective indicators in Triton X-100 neutral micelles (from Fernandez and Fromherz (39)). Anionic liposomes containing sodium oleyl sulfate (SOS) and cholesterol (50 mole % cholesterol, 40 mM total lipids ▲, △; anionic liposomes containing SOS, cholesterol and oleic acid (40 mole % oleic acid, 40 mole % cholesterol, 20 mole % SOS, 50 mM total lipid ■, □; and the extracellular vesicles ●, ○.

SURFACE pH PROBES

	PROTONATED FORM (Lacks Fluorescence)		DEPROTONATED FORM (Fluoresces)	pH RANGE
Hydroxy Coumarin			 (Charged)	pH 7-11
Amino Coumarin	 (Charged)			pH 1-4

The uncharged form has a preference for uncharged surfaces such as Triton X-100 or PC (Phospharidyl Choline)

TABLE VII

Alkylated pH Indicator Studies on the Extracellular Vesicles

Table: A

pH	% Dissocation
11.85	100%
11.70	100
11.50	98.4
11.11	96.7
10.50	88.3
9.55	46.7
8.70	15.0
6.40	0.0

The Alkylated hydroxycoumarin indicator in 3 ml of an extracellular vesicle (ECV) dispersion was titrated with HCl. The percent dissociation is based upon fluoresence quenching. Percentages are normalized to the maximum fluorescent yield of the unprotonated indicator.

Table: B

pH	% Dissociation
6.60	100.0
5.15	100.0
3.45	100.0
2.70	100.0
2.40	97.2
2.20	92.4
1.95	86.4
1.65	79.7
1.40	71.0
1.20	63.7
1.00	53.8
.80	49.0
less than 0*	0

The alkylated aminocoumarin indicator was titrated and assayed as in Table A.

*500 ml of 10N HCl solution was added and the fluorescence was totally quenched. the pH was not measured in this sample.

DISCUSSION

Preparation and Composition of Extracellular vesicles

Although many attempts have been made to isolate the plasma membrane of O. danica, all of these attempts have been unsuccessful. Most of the early membrane preparations were mixtures of organelle membranes. In retrospect the detergent lipids of the membrane appeared to prevent the isolation of pure plasma membrane. Chen et al. (31) sought to obtain pure plasma membrane by first isolating the flagella, an organelle that contained a single membrane. The flagellar membrane is continuous with the plasma membrane so that it may be considered an extension of it. This isolation resulted in the discovery that the flagellar membrane of O. danica lacks phospholipids and is constituted primarily of chlorosulfolipids (25,31).

The phytoflagellate has been shown to excrete copious amount of vesicles (50,51) into its culture media. It seemed reasonable to examine these vesicles because the possibility existed that they may reflect the lipid composition of the plasma membrane and thus provide material for structural studies on the membrane. On the other hand it was possible that the process of blebbing selectively alters the composition and the blebs may therefore be unique in their lipid composition compared to that of the plasma membrane. A simple and rapid procedure was developed to isolate the ECV by using a Millipore cassette system. This system was an important improvement over differential centrifugation because large amounts of excreted membrane could be obtained in a few hours. The results suggest

that the Millipore filter alters the membrane by both adsorbing chlorosulfolipids and by enhancing their hydrolysis during isolation. For [illegible]eason a second and gentler isolation procedure based on differential centrifugation (Preparation II) was undertaken. The data show that reduced levels of chlorosulfolipid are present in membrane isolated by using the Millipore method (Preparation I). The samples were examined under electron microscopy and found to be free of other cellular contamination. Furthermore, the trilamellar vesicles obtained in this manner are of relatively uniform size. This may in part be due to sonication. Membrane prepared by centrifugation and without sonication (Preparation II) is less uniform in size and less pure as a membrane preparation.

O. danica has neither a cell wall nor an outer membrane (5,107-110). Furthermore, it does not contain phospholipids in its flagellar membrane. The existence of unusual lipids in an outer membrane that is not protected by a wall may be a common feature of protozoa lacking a wall or peptidoglycan. This introduces two interesting problems for the organism's survival. The first is its resistance to osmotic pressure and the second is its resistance to extracellular enzyme attack from other organisms. Thus, phosphonolipids and other unique lipid structures dominate the surface membranes and cilia of Tetrahymena (26) and Paramecium (29). It has been suggested that such lipids are resistant to phospholipases (26,27).

The flagellar membrane may be much the same in its lipid composition as the plasma membrane since the membranes are continuous.

Both membranes lack a protective wall and yet are presumably phospholipase resistant. It was also reasonable to expect that the extracellular vesicles might contain the same chlorosulfolipids since they were derived (by blebbing) from the flagellar membrane and also from the plasma membrane. Thus the plasma membrane of the cell could be indirectly examined to establish if it is likewise composed of these unusual lipids.

The lipid composition of the ECV reported herein was essentially that of the flagellar membrane (25). The most outstanding feature about the membrane lipid composition is the complete absence of phospholipids and their replacement as the dominant polar lipid by the chlorsulfolipids (70% total lipid; > 90% polar lipid). The mere absence of phospholipids does not necessarily have important implications to a unique membrane structure as these polar lipids may be replaced by other ionic or polar lipids with analogous structure. This has previously been noted in O. danica by Elovson (49), who identified 1(3),-2-diacylglycerol-3(1)-0-4-(N,N,N trimethyl) homoserine as one of the major lipids in the phytoflagellate. The compound has steric and ionic-hydrophobic structural properties similar to those of the phospholipids. This lipid is virtually absent from the flagellar membrane and from the extracellular vesicles.

Slightly more unknown polar lipids (8.8%) were found in the ECV compared to those (5.3%) of the flagellar membrane. The non-polar lipid composition of the ECV (22.%) is approximately the same as that of the flagellar membrane. It is unusual to find free fatty

acids in bilayers, but in the case of flagellar membrane of O. danica a substantial portion of the total lipids is free fatty acids. This has also been found in the ECV wherein about 14% of the total lipid is free fatty acids. These appear not to be due to formation as a result of either lipase activity or hydrolysis during isolation (25).

The flagellar membrane consists of five major proteins having apparent molecular weight of 54,000, 47,000, 34,000, 31,000 and 28,000 daltons. The mastigonemes of which 17% (w/w) is carbohydrate consists of three glycoproteins of apparent molecular weight 110,000, 83,000, 54,000, and low molecular weight oligosaccharide (31). In the case of the ECV the proteins (14-18%) consists of 4 major components (apparent molecular weights, 97,000, 85,000, 47,000 and 19,000) and one minor protein (apparent molecular weight of 110,000). All protein components were shown by periodic-acid Schiff reagent to be glycoproteins. The membrane preparations of two protozoa Entamoeba invadens (111) and Acanthamoeba castellanii (112) contain a 45,000 dalton protein. This protein has been shown in a A. castellanii membrane preparation to be actin by several criteria. Korn et al.(112) found that the appearance of this band on SDS-PAGE is correlated with membrane associated filaments in the electron micrographs.

In the flagella membrane of O. danica and the ECV there is a 47,000 dalton protein. The latter protein could conceivably be actin, but this would seem unlikely because actin is not a glycoprotein and it is not a likely excretory product.

It should be noted that the supernatant that was in equilibrium

with the sonicated ECV (Preparation I) gave an identical electrophoretic pattern which indicates that the membrane solubilized by sonication of the ECV has the same protein composition. This implies that selected proteins (such as extrinsic) are removed from the membrane whereas other (intrinsic) are retained. Frequently sonication releases proteins that are not identified with membrane integrity. The data implies that the supernatant either contains membrane proteins solubilized by the detergent lipid or exceedingly small vesicles that are not collected in the centrifuge.

The sugar analysis of the flagellar membrane showed that carbohydrate constitutes only 1% of its total dry weight (32). It consists primarily of six sugars: rhamnose, N-acetyl glucosamine, fucose, xylose, mannose and galactose. Rhamnose is present in the highest mole present. Glucose had been found in the preparation as an artifact. It had been found that the free glucose (a medium component) was entrapped in the flagellar membrane vesicles (36) during their isolation. The flagellar membrane contained a large amount (47.5%) of an unknown which charred during anthrone analysis. This prevented the use of anthrone as a quantitative measure of the sugar composition.

In the ECV, carbohydrate was the major component (35%) and anthrone was useful for its estimation. After hydrolysis of the oligosaccharides it was possible to examine the sugar composition by TLC. The same sugars were found in the ECV as were found in the flagellar membrane, although mannose is present in the ECV in the highest mole precent rather than rhamnose. The sugars are not free sugars but oligosaccharides (most probably a glycoproteins) as they were not

dialysable. It is reasonable to say that the carbohydrate composition of the ECV is different from that of the flagella membrane. It seems that the flagellar membrane has a carbohydrate composition that may be reduced to simple whole numbers of relative amounts of its sugar constituents suggesting a repeating oligosaccharide. The ECV is likely to have many oligosaccharide types.

The oligosaccharides are presumably covalently bound to protein. They would not be expected to be part of the hydrophobic domain of the bilayer, this is important because the hydrophobic domain of the bilayer appears to be unique in this system and the membrane's composition is dominantly carbohydrate. Thus the lipids, which are less than 30% of the membrane including the carbohydrate, would be approximately half of the membrane if the carbohydrate were not present.

Assuming that the formation of the ECV by "blebbing" or exocytosis occurs with retention of configuration as it does in all other systems that have been studied and assuming that the glycoconjugates are asymmetrically arranged on the bilayer, then they will face the exterior face of the ECV bilayer.

Studies on the pH at the Membrane Surface:

The distribution of negative sites on the cell surface of [illegible] has been studied by electron microscopy with the use of colloidal iron as the visual marker (113). Danon et al. (114) introduced the use of polycationic ferritin (PCF) for localizing anionic sites in electron micrographs.

Stern et al. (36) used PCF for studying the localization of anionic charges on the exterior surface of the plasma and flagellar membranes. They suggested that the chlorosulfatides are partially buried under peripheral proteins, glycoproteins, polysaccharides or a unique, invisible (in electron microscopy), polymeric coat on the cell surface. This suggestion was based on the observation that the membrane was stained with heavy metal cations but not with the 10.0 nm PCF particles. This would explain a low density of PCF binding to the plasma and flagellar membrane. Their data suggested that the process of blebbing is associated with a breakdown of a hypothetical "polymeric coating" because PCF exhibited access to the ECV membrane but not to the plasma membrane from which the ECV was derived. Thus vesicles excreted by the cell show a high density of PCF binding. These electron micrographs are hard to interpret because the ferritin is destroyed by the detergent lipids and the membrane is destroyed by the cationized ferritin when mutual contact is made.

Stern et al. (36) found unknown material in flagellar membrane (about 47.5% by weight), this unknown contains (29.3% C, 2.42% H and 16.9% O) and is not consistent with neutral sugar or amino sugar.

They suggested that this unknown material may be a polymeric coat. A coat is postulated because the lipid and protein composition (negatively charged lipids and the negatively charged or acidic proteins) would necessarily bind the PCF. The low PCF binding was interpreted as evidence for a protective uncharged coat. Our results on the ECV indicate that such a "polymeric coat" is absent from, or present in much lower concentration in, this preparation. It is reasonable to speculate that the formation of the vesicles is associated with a cleavage of this postulated polymeric coat.

Fromherz (39) introduced the use of two alkylated pH indicators to measure the pH at charged and uncharged surfaces. The indicators were successfully used to study the pH at the surfaces of negatively charged micelles and monolayers in which the observations conformed with the Gouy-Chapman theory. Fernandez (39) has extended these studies to PC bilayers. Heller et al. (37) have further extended the use of these indicators to alkyl sulfate model systems consisting of a liposome dispersion containing alkyl sulfates and alkanols. One such model system had a molar ratio of 40% cholesterol, 40% oleic acid and 20% oleyl sulfate. Studies on this model system (Fig. 13) showed that the hydroxycoumarin indicator displayed a pK shift of 1.7 pH units above its value for the neutral system (Triton X-100) whereas, no shift was found for the aminocoumarin indicator. The latter is used to examine the lower pH (0-4) range while the hydroxycoumarin is useful in the upper pH range of 6-12.

There is qualitative agreement of the fatty-acid-containing model system with the natural ECV system. The correspondence can

be rationalized by noting that at high pH the carboxylates are known to be deprotonated (charged) whereas in the low pH range they are protonated (uncharged). Thus a pK shift of the alkylated indicators in the fatty-acid-containing model system compared to that of the SDS model system that lacks fatty acids is explained by the "polarity effect" of less surface charge at low pH. This may be taken as further evidence that the fatty acids in the lipid compositional studies are present as free fatty acids in the membrane, they are not an artifact produced a lipase during lipid extraction.

Obviously at high pH the surface has a greater charge density than in the low pH region. The low pH indicator shows a shift of only 0.45 pH units below its observed pK in the neutral Triton X-100 membrane. A small shift at low pH beyond the neutral state might perhaps be rationalized by considering that the surface contains a positively charged component since cationic surfaces cause shifts to lower pK's (39). However, both in the fatty-acid-containing model system and the ECV membrane exhibit similar properties at low pH. Since this model membrane contains no cation species but exhibit qualititive similar shift of the indicator to the ECV membrane system it appears that this low shift must have some other explanation. It was previously shown that at low pH, fatty acid-liposomes forms lipid oil droplets (37), the effect of such droplets on the indicator's pK has not been investigated.

Studies on Membrane Stability

It has been shown by Stern et al. (36) that the flagellar membrane of O. danica maintains its integrity without any divalent metal cations. These investigators also showed that the membrane's nitrogen is completely accounted for by its protein and that the protein is acidic. This excluded the possibility of an organic cation stabilizing the secondary sulfate in the hydrophobic domain of the bilayer.

The secondary sulfate esters, which are suspected to be deep in the bilayer may remain so if they are in the protonated form. Since all other possibilities for a counterion appear to have been ruled out we are forced to consider the notion that protonated secondary sulfates might be present in the hydrophobic region. Alkyl sulfates are exceedingly stable in base whereas acid not only hydrolyses them but the reaction is autocatalytic since it produces sulfuric acid.

Mayers and Haines (12) had earlier developed a solvolysis procedure which hydrolyses alkyl sulfates selectively in a few minutes in moist dioxane with retention of configuration of the secondary C-O bond. Hydrolysis in strong acid (aqueous) generally takes hours.

Haines (14) suggests that the sulfate groups must be protected from nucleophiles and from protonation of the C-O-S oxygen in organic solvents. Such protection may be provided by water, protons as positively charged species enter the low dielectric with difficulty, so the possibility that the membrane is actually held together by extreme acidity in combination with its stabilization in the

chlorinated alkane, which acts like a halocarbon solvent, has been raised.

It should be noted that the structures of the chlorinated sulfatides (Fig. 2) show that the sulfate esters are surrounded by chloro groups on the chains. These groups affect the solvation of polar compounds in halocarbon solvents and the existence in a bilayer of a "belt" of halocarbon at the level of the secondary sulfates (assuming that they are in the hydrophobic region of the bilayer) must be considered.

To study the membrane stability we examined the effect of pH and of sonication on the quality of the membrane preparation. In the course of our studies we noticed that loss of membrane occurred with sonication at high pH. It also appears that the solubilizations of sulfatide is accompanied by release of protons from the membrane on a 1:1 basis which lends support to the notion that the membrane harbors protons. It releases them in a molar ratio with chlorosulfolipid from the bilayer. In the case of sonicated membrane greater release of sulfatide into the supernatant is accompanied by the release of fewer protons. Electrophoresis of the supernatant obtained from sonication shows the same protein electrophoretic pattern as the membrane implying that sonication results in the formation of lower molecular weight aggregates, which aggregates are not collected during centrifugation. All of this suggest that the alkyl disulfate membrane is held together by hydrogen bonds involving acidic protons.

This membrane appears to be a typical acid membrane (acid membranes contain anionic and uncharged lipids, but lack zwitterionic lipids) in that the proteins are also acidic. A well known example is the purple membrane which contains the acid protein, bacteriorhodopsin (80). Chloroplasts are likewise constituted of acidic lipids and contain acidic proteins.

It might be noted that each of the membranes made exclusively of anionic lipids have also been found to be proton pumping membranes. This does not mean all proton pumping membranes are made only of acid lipids (e.g., mitochrondria, E. coli). However, the latter do contain acidic lipids and in some cases the acidic lipids are specifically identified with proton pumping proteins (e.g., cardiolipin and cytochrome oxidase).

Studies of Fatty Acid Removal by Bovine Serum Albumin (BSA):

Studies on the ECV preparation with bovine serum albumin (BSA) presented further evidence for the presence of free fatty acids in the natural ECV membrane. The depletion of membrane free fatty acids with a high concentration of albumin appears to disrupt the membrane. It is presumed that the denaturation of the albumin results from the release of chlorosulfolipids from the membrane.

Myelin formation in the presence of moderate amounts of albumin is another indication that the removal of free fatty acids from the membrane has an impact on the membrane's structure. This may be taken as an indication that the fatty acids play an important structural role in the membrane.

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