ISOLATION AND PARTIAL CHARACTERIZATION OF FLAGELLA MEMBRANE OF *OCHROMONAS DANICA*

by

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SUMMARY

The isolation and purification of the flagella membrane of the phytoflagellate, Ochromonas danica, is described. The procedure was simple, mild, rapid, and it produced a pure membrane preparation. Additionally, methods were developed for the isolation of clean preparations of most of the components of Ochromonas danica flagella from a single flagella preparation, namely: mastigonemes, axonemes (9+2), "extramastigoneme filaments" and flagella membrane. Each component was studied by electron microscopy and acrylamide gel electrophoresis. Some qualitative and quantitative studies were done on the lipids of flagella membrane, flagella and whole cell (dark grown cultures). The isolated flagella preparation was nearly free of other cellular organelles as judged by phase contrast and electron microscopy. The purified membrane preparation consistedof small vesicles (500 - 1500Å in diameter) with a trilamellar pattern about 80Å thick. Isolated membrane was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, displaying 5 major protein bands, 5 minor protein bands and some protein which remained at the origin. The 5 major protein components had molecular weights of 54,000, 47,000, 34,500, 31,000 and 28,000 daltons. Other flagellar fractions showed characteristic patterns on analysis by SDS-gel electrophoresis. All mastigoneme protein components are glycoproteins as judged by periodic acid Schiff staining.

The lipids of the whole falgella and the flagella membrane preparation were isolated and compared with those of the whole cell. The polar lipids were separated by two dimensional thin layer chromatography. One dimensional thin layer chromatography was used for the separation of the nonpolar lipids. In all respects the lipids of the whole falgella were identical to those of the flagella membrane. These methods established the presence in flagella membrane of the polychlorosulfolipids of O. danica as 73 molar percent of the total polar lipids, these sulfolipids had been previously characterized as 1,14-docosanediol-1,14-disulfate and 1,15-tetracosanediol-1,15-disulfate containing zero to six chloro groups substituting for hydrogen on the chain. In addition to six unknown polar lipids one lipid is present which has recently been identified as 1(3),2-diacylglyceryl-3(1)-O-4'-(N,N,N-trimethyl)-homoserine. It constituted 9 molar percent of the polar lipids. Both phosphorus analysis on each lipid and the molybdenum spray reagent for phospholipids on the chromatogram showed that there is no phospholipid present in O. danica flagellar membrane. Positive reactions to the diphenylamine spray reagent suggest that up to 4 of the unknown polar lipids are glycolipids. Of these, three reacted positively with ninhydrin. All of the unknown lipids reacted with the acidified 2,4-dinitrophenylhydrazine spray reagent suggesting the presence of aldehyde, ketone, glycoside or plasmalogen.

One unknown substance (near the origin) appears to be not a lipid but a "glycan". It showed a positive reaction with Dragendorff reagent suggesting the presence of a quarternary amine group. This same unknown spot is not synthesized from [1-^{14}C]acetate under the growth conditions used as revealed by autoradiograms of thin layer chromatograms. It contained 35% hexose or hexosamine. The "glycan" represents 62% of the lipid extract. It is devoid of phosphorous (<0.07%) and is less than 4% protein (or phenol or peptide) as judged by the Lowry assay using bovine serum albumin as a standard.

Analysis of the nonpolar lipids of the flagella membrane indicated a surprisingly large amount of free fatty acids (42% of the total lipids). These free fatty acids could be the true components of the membrane or artifacts of the extraction procedure although every precaution was taken to prevent artifactual production of free fatty acids. The sterols constitute 29% of the total lipids. Sterol esters were absent from the membrane. There are 2 additional major unknown nonpolar lipids present.

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

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INTRODUCTION

Biological membranes play a crucial role in almost all cellular activity. Insights are slowly evolving about their structure and about the mechanisms of membrane associated reactions. Protozoa, being unicellular, possess the same advantages found in bacteria with respect to ease of experimental manipulation. Yet they strongly resemble the cells of higher animals and plants in their diversity of membrane rich organelles. The cytoplasmic membrane of many protozoa, devoid of cell wall, is the only outer barrier of the cell. Many major activities of the cell, which occur in, on or through the cell membrane, are similar to mammalian cells. Protozoa have proven to be a useful model system for studying membrane structure and biogenesis. The phytoflagellate Ochromonas danica, in addition to these advantages contain a substantial quantity of a series of unusual sulfolipids, making an even more attractive model system to study the structure of membranes.

Basic understanding of membrane structure began in 1895 (1) with the observation that certain anesthetic substances that were lipoidal in nature probably acted by dissolving in or interacting with the membrane. This observation defined the lipid nature of the membrane. Sometime later Gorter and Grendel (2) reported that the lipid of erythrocyte provided just enough surface area to cover the cell twice if the lipids were oriented radial to the spherical cell and hence suggested that the membrane existed as a lipid bilayer. Their data

were later reexamined and reconsidered. The use of newer techniques have continued to support their proposal of a lipid bilayer as a basis for membrane structure. The experiments of Gorter and Grendel were followed by Danielle, Harvey and Davson. The formal presentation of the model was given in 1935 and this representation is known as the Danielli-Davson model (3). On the basis of new observations Robertson in 1957 (4) modified the Danielli-Davson model and proposed that the structure, which he termed the unit membrane, represented the universal structure for all biological membranes. The unit membrane hypothesis enjoyed almost universal acceptance for several years. However questions were raised in the early 1960's (5, 6, 7, 8) when several electron micrographs revealed appearance of subunits in the plane of the membrane. Subsequent analysis has frequently produced other explanations for these subunit observations. Firm evidence for these proposals have generally not been forthcoming.

Freeze-cleavage techniques (9) have shown the existence of granular material inside of a continuous bilayer sandwich. There are many more specific proposals for membrane ultrastructure and many membrane models put forward; these have been reviewed in the literature (5, 10, 11).

Among all the models proposed, the most plausible one is the fluid mosaic model proposed by Singer and Nicolson (12). These

investigators suggest membrane-bound proteins are of two types "peripheral" and "integral". The peripheral proteins are readily removed by mild treatment; the integral proteins are difficult to remove. The latter proteins have an α-helical content in the membrane of about 40%; there is little of no β-structure. The high α-helical content is attributed to extensive hydrogen bonding induced by the presence of substantial, tightly packed, non-polar regions. This suggests that the integral proteins in intack membranes are largely globular in shape rather than spread out as monolayers. A model therefore, in which globular protein molecules are intercalated within the membrane would be more satisfactory.

Phase transition induced by heat corresponding to the "melting" of the hydrocarbon chains with a bilayer configuration have been demonstrated in dry phospholipids, phospholipid-water mixtures and in intact mycoplasma membrane (13,14). Above the transition temperature much of the lipid in a biomembrane is mobile. Lateral diffusion of lipid in a lipid bilayer is very rapid, lipid diffusion between the bilayers is extremely slow (15). In the fluid-mosaic model, the proteins that are integral to the membrane are a heterogeneous set of globular molecules, each arranged in an amphipathic structure, that is, with the ionic and highly polar groups protruding from the membrane into the aqueous phases, and the nonpolar groups largely buried in the hydrophobic interior of the membrane. These globular molecules are

partially embedded in a matrix of phospholipid. The bulk of the lipid is organized as a discontinuous, fluid bilayer, although a small fraction of the lipid may interact specifically with the membrane proteins.

Bretscher (10) presented a picture of the erythrocyte membrane which is based on a lipid bilayer having compositional asymmetry in its phospholipid and glycolipid components with choline phospholipids and glycolipids in the external half and amino phospholipids in the cytoplasmic half. A major protein and glycoprotein are located in a fixed orientation across the membrane, and many more proteins are associated with the inner surface of the bilayer. Labeling techniques have shown that the mitochondrial inner membrane is organized in a similar fashion with an asymmetric organization. ATPase is located exclusively on the inner side of the membrane and the cytochrome C molecules are found only on the outer surface (16).

The flagella of eucaryotic cells consist of a bundle of microtubules, arranged in 9+2 pattern, called an axoneme, embedded in a matrix and surrounded by membrane. Attached to the membrane are extraflagellar hairs, called the mastigonemes. A sketch of Ochromonas danica flagellum is shown in Fig. 1. The mastigonemes of O. danica are of two structural types, fibrous and tubular. There are in addition two sets of lateral filaments along the entire tubular mastigoneme shaft (17,18). In contrast Chlamydomonas mastigonemes are of only one structural type and each

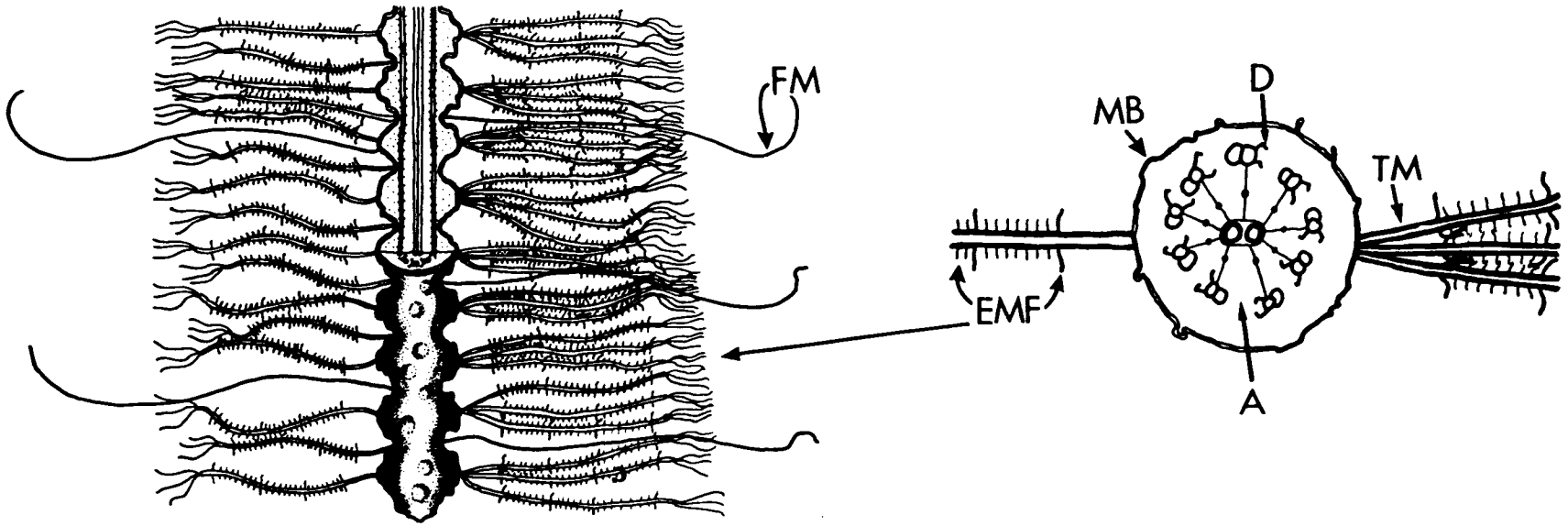

FM
MB
D
TM
EMF
A

Fig. 1. Diagrammatic representation of flagella ultrastructure.

MB: Flagella membrane

D: Dyneins

A: (9+2) Axoneme

TM: Tubular mastigonemes

FM: Fibrous mastigonemes

EMF: Extramastigoneme filaments

mastigoneme appears to be composed of a single row of subunits. Electrophoretic analysis of Chlamydomonas mastigonemes indicates that they contain a single glycoprotein of about 170,000 daltons (19).

O. danica moves by means of a planar sine wave in the long anteriorly-directed flagellum. The wave starts at the basal end of the flagellum and progresses distally. The organisms are pulled forward by the action of the long flagellum. (O. danica has a long and a shart flagella). Jahn 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 (20). It has been found that the ATP hydrolysis is localized to the nine outer microtubules and specifically to the arms on these microtubules (21). These arms are proteins and are called dyneins. Dyneins of several species have been studied and found to have a molecular weight of about 500,000 daltons (23). The ciliary motility and a "sliding filament" model has been reviewed (22). The fine structure of the outer doublet microtubules of cilia and flagella has been investigated (24, 25) and a model has been proposed that the outer doublet contains a total of 23 protofilaments (or subunits as observed in cross section), arranged as illustrated in Fig. 2. Two tubulins (tubulins 1 and 2) are present in both A and B tubules of the outer doublets of Chlamydomonas (25). Tubulins 1 and 2 were shown to have apparent molecular weights of 56,000 and 53,000 daltons respectively (19) in the Chlamydomonas flagella.

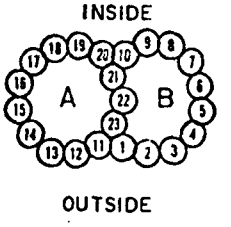


Fig. 2. The arrangement of the 23 protofilaments of the outer doublet microtubules of flagella.

Our interest in the isolation of flagella membrane stems from three considerations. First, many continuities between different membrane systems have been reported (26). To obtain a pure cytolasmic membrane preparation, free from contamination of the quantitatively more abundant intracellular membrane is almost impossible because organelles appear to fragment easily placing limitations on the present isolation methods. There also seems to be the problem of release of intracellular glycosidase and hydrolytic enzymes upon cell rupture which may result in either loss from or adsorption to the plasma membrane of complex carbohydrate species. On the other hand, flagella, which is devoid of any internal membrane systems, may be readily detached and isolated by gentle differential centrifugation. Such a procedure would allow us to obtain a very pure membrane preparation free from contamination by any other membrane. Second, the problem of membrane structure must be considered in relation to the problems of membrane function and membrane biosynthesis. Flagella may be amputated and the synchrony of regeneration which follows may provide us with a handle to approach the problem of organelle development and membrane biogenesis (27). It has already been noted that the "new" membrane of the growing flagella is not at the base of the flagella but at the distal end (27). A remarkable observation in view of the fact that protein synthesis occurs exclusively in the cell body. Third, the flagella membrane has two interesting aspects. It is on the one hand, contiguous with the cell membrane of all

organisms in which it has been studied and yet it is a component of a specialized organelle. It is suggested that the constituents of the flagella are transmitted as a cistron genetically since so many protein qualities are so similar from species to species. The surface membrane of these species however, vary widely. It is likely, therefore, that the flagella membrane, which serves as a cell surface membrane on the exterior and possibly in gamete recognition and agglutination (18,28) is also a functionary of some sort in flagellar action. In the case of Ochromonas danica this bimodal function makes the system especially amenable to structural studies since the unique lipids of the membrane have characteristics which can be exploited by physical techniques. A good example of this approach rests in the presence of large amounts of the halosulfolipids in the phytoflagellate. These substances are derivatives of 1,14-docosanediol-1,14-disulfate and 1,15-tetracosanediol-1,15-disulfate with from 0 to 6 chlorine atoms replacing hydrogens on the chain (Fig. 3) (29,30,31,32,33,34,35). These substances are amenable to X-ray studies on the natural membrane because of the presence of electron dense atoms in their structure. The arrangement of these molecules in the membrane will be of significance to membrane structure since the substances are the only known lipids that do not have all their polar or ionic groups at one end of the molecule.

Chlorosulfolipids of Ochromonas danica

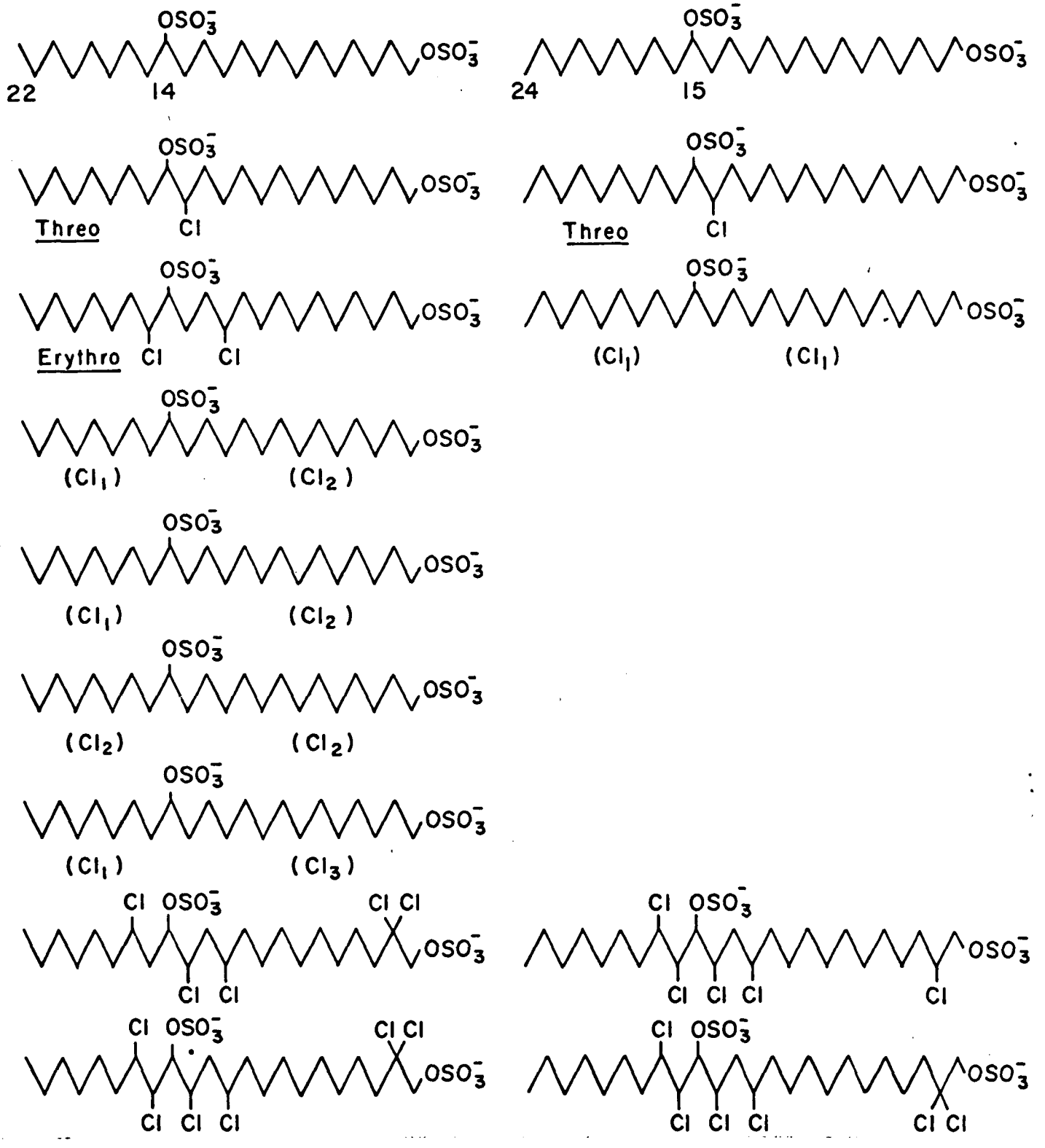


Fig.3. The ahalo, monochloro and hexachloro derivatives of 1,14-docosanediol-1,14-disulfate are the dominante structures.

EXPERIMENTAL PROCEDURE

Cultures - Ochromonas danica was grown in the chemically defined medium of Aaronson and Baker (36) at 23° in darkness. Inoculations were conducted in ambient light. Cells were cultured in tubes or flasks wrapped in aluminum foil, placed in light tight boxes.

Incubations - Sodium [1-^{14}C] acetate (specific activity 1 mCi/1.4 mg, total 4 mCi) dissolved in ethanol were added to a 3-day culture. The final concentration of ethanol in media was 0.3% (v/v). Cultures were maintained in a dry box connected to three successive traps containing 1 N NaOH.

Flagella Detachment and Isolation (Scheme I) - Cultures of O. danica were harvested 5 days after inoculation by centrifugation for 15 min at 4° and 300 X g in a Sovall RC2-B centrifuge and were washed with fresh media twice. The cells were then resuspended in fresh media (1/30 of the original culture volume) at 4° and cooled for 1 hour before deflagellation. Flagella were detached by agitation in a centrifuge tube for 24 sec (3 sec each time, 8 times), at top speed in a Vari-Whirl mixer (Van Water & Rogers Scientific). Intact and deflagellated cell bodies were then removed from the medium by centrifugation for 10 min at 208 X g at 4° three times to remove the residue cell bodies. The remaining supernation which contained flagella was centrifuged at 13,300 X g for 20 min in an SS-34 rotor of Sorvall RC2-B Centrifuge. The pellet obtained was milky white and appeared to be pure flagella under phase contrast and electron microscopy.

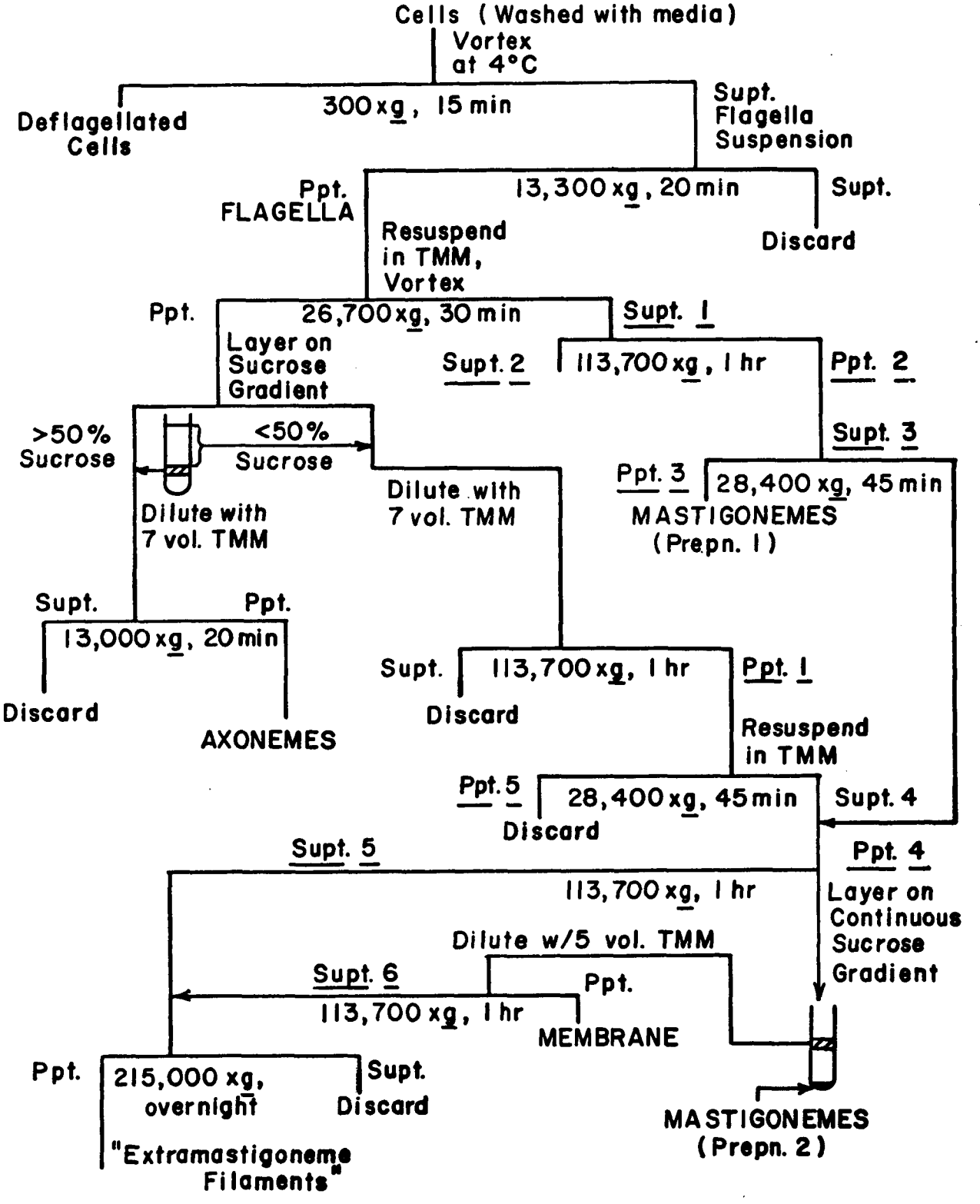
Fractionation of Flagella - Flagella pellets were resuspended in 50-100 volumes of TMM[1] (10 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mM mercaptoethanol) mixed at top speed in a vortex mixer for 15 min, stirred overnight to detach the membrane from the axonemes matrix and mastigonemes. The solution was centrifuged at 26,700 X g for 30 min. Small membrane vesicles, mastigonemes, and "extramastigoneme filaments" appeared in the supernant (Scheme I, Supt. 1). Large pieces of membrane were present in the pellet together with the axonemes[2].

Isolation of Axonemes - The above pellet was thoroughly resuspended in 5 to 10 ml of TMM and was layered over a discontinuous sucrose gradient which was made of 6 ml each of 60%, 50%, 45% and 40% sucrose solutions (w/v) in the TMM buffer. They were centrifuged at 41,400 X g for 4 hours in an SW 25.1 rotor in a Model L Ultracentrifuge. The axonemes banded at the interface of 50% and 60% sucrose while the membrane and some mastigonemes were suspended in solution above 50% sucrose layer. The membrane and mastigonemes were then collected, diluted with 7 volumes of TMM and centrifuged at 113,700 X g for 1 hour. The pellet (Scheme I, PPT 1) contained membrane vesicles and mastigonemes. The axonemes band was collected, diluted, and centrifuged at 13,000 X g for 20 min. The pellet constitutes a preparation of axonemes.

[1] The abbreviation use are: TMM, 10 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mM mercaptoethanol; SDS, sodium dodecyl sulfate.

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

SCHEME I


Cells (Washed with media)
Vortex at 4°C
300 x g, 15 min
Deflagellated Cells
Supt. Flagella Suspension
13,300 x g, 20 min
Ppt. FLAGELLA
Supt.
Discard
Resuspend in TMM, Vortex
26,700 x g, 30 min
Ppt.
Supt. 1
Layer on Sucrose Gradient
Supt. 2
113,700 x g, 1 hr
Ppt. 2
Supt. 3
>50% Sucrose
<50% Sucrose
Dilute with 7 vol. TMM
Dilute with 7 vol. TMM
Ppt. 3
28,400 x g, 45 min
MASTIGONEMES (Prepn. 1)
Supt.
Ppt.
13,000 x g, 20 min
Discard
AXONEMES
Supt.
113,700 x g, 1 hr
Ppt. 1
Discard
Resuspend in TMM
Ppt. 5
28,400 x g, 45 min
Supt. 4
Discard
Supt. 5
Ppt. 4
113,700 x g, 1 hr
Layer on Continuous Sucrose Gradient
Dilute w/5 vol. TMM
Supt. 6
Ppt.
113,700 x g, 1 hr
MEMBRANE
Ppt.
215,000 x g, overnight
Supt.
Discard
"Extramastigoneme Filaments"
MASTIGONEMES (Prepn. 2)

Isolation of Mastigonemes - A centrifugation force of 113,700 X g was found to convert some membrane sheets and large vesicles into small vesicles (approx. 1,000 Å in diameter). This conversion apparently allowed the membrane to sediment at a speed different from that of the mastigonemes. Supt. 1 (Scheme I) was therefore centrifuged at 113,700 X g for 1 hour. The precipitate (Scheme I, PPT. 2) was a mixture of mastigonemes and membrane vesicles. This pellet was then resuspended in the same buffer and centrifuged at 28,400 X g for 45 min to precipitate the mastigonemes (Scheme I, PPT.3). The supernatant (Scheme I, Supt. 3) consisted primarily of membrane vesicles with some contamination of mastigonemes and "extramastigoneme filaments".

Isolation of Flagella Membrane - The membrane pellet (Scheme I, PPT I) was resuspended in TMM buffer and centrifuged at 28,400 X g for 45 min. The supernatant (Scheme I, Supt. 4) was combined with Supt. 3 (Scheme I) and centrifuged at 113,700 X g for 1 hour. The supernatant (Scheme I, Supt. 5) contained "extramastigoneme filaments". The membrane pellet (Scheme I, PPT 4) was resuspended in 5 to 10 ml of TMM buffer and layered over a linear 8.6% to 60% sucrose gradient in TMM buffer and centrifuged at 41,400 X g (Spinco SW 25.1 rotor) for 16 hours. The membrane banded at density between 1.11 and 1.15. The precipitate at the bottom of the tube contained mastigonemes and some membrane contamination (mastigonemes Prepn. 2). The membrane band was collected, diluted and centrifuged at 113,700 X g for 1 hour. The precipitate was pure membrane. The supernatant (Scheme I, Supt. 6) contained "extramastigoneme filaments".

Isolation of "Extramastigoneme Filaments" - Supt. 5 and Supt. 6 (Scheme I) were combined and centrifuged first at 113,700 X g for 2 hours to remove any residue membrane vesicles and then at 215,000 X g overnight. The "extramastigoneme filaments" appeared as a pellet.

Electron Microscopy - All flagella fractions obtained as pellets were fixed (for a period which varied from 2 to 16 hours) with 2% glutaraldehyde (w/v) in 0.2 M cacodylate buffer, pH 7.0, at 4°, and post-fixed for a period which varied from 2 to 16 hours in 1.0% (w/v) osmium tetraoxide prepared in 0.2 M cacodylate buffer, pH 7.0, at 4° in the same buffer. The pellets were sequentially dehydrated with 50, 75, 95, and 100% (v/v) ethanol, cleared in propylene oxide and infiltrated overnight with a mixture of Epon 812 and propylene oxide 1:1 (v/v), and embedded in Epon resin. The pellets were then sectioned with a Porter Blum Ultramicrotome (MT-2). The sections were supported on carbon reinforced collodion covered grids and post-stained with lead citrate (37) and observed with a Philips 300 electron microscope.

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

Light Microscopy - Phase contrast light microscopy was conducted with a Wild Heerbrugg M 20 91257 Microscope.

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (38) - (A), Preparation of protein solutions: The proteins were incubated at 37° for 2 hours in 0.01 M sodium phosphate buffer, pH 7.0, 1% in SDS and 1% in β-mercaptoethanol prior to electrophoresis. The protein concentration was normally 0.5 mg per ml. (B), Preparation of gels: Gel buffer contained 7.8 g $NaH_2PO_4 \cdot H_2O$, 38.6 g of $Na_2HPO_4 \cdot 7H_2O$, and 2 g of SDS per liter. The acrylamide solution consisted of 22.2 g of acrylamide and 0.6 g of methylenebisacrylamide dissolved in water to give 100 ml of solution. For a typical run of twelve 10% (w/v) acrylamide gels, 15 ml of gel buffer were deaerated and mixed with 13.5 ml of acrylamide solution. After further deaeration, 1.5 ml of freshly made ammonium persulfate solution (15 mg per ml) and 0.045 ml of N,N,N',N'-tetramethyl-ethylenediamine were added. After mixing, each tube was filled with about 2 ml of the solution. Before the gel hardened a few drops of water were layered on top of the gel solution. After 20 min an interface could be seen indicating that the gel had solidified. (C), Preparation of samples: Onto each gel was placed 3 µl of tracking dye (0.05% bromphenol blue in water) 1 drop of glycerol, 5 µl of mercaptoethanol, and 50 µl of 0.01 M sodium phosphate buffer, pH 7.0, 1% (w/v) SDS, and 1% (w/v) mercaptoethanol. Fifteen to 50 µg of the protein solution were added. Gel buffer, diluted 1:1 with water, was carefully layered on top of each sample to fill the tubes. The two compartments of the electrophoresis apparatus were filled with gel buffer, diluted 1:1 with water. Electrophoresis was performed at a constant current of 8 ma per gel for 4 to 4.5

hours. After electrophoresis, the gels were removed by a syringe and by using a pipette bulb to exert pressure. The distance moved by the tracking dye was marked on the gels. (D), Staining and destaining: The gels were placed in tubes filled with staining solution prepared by dissolving 1.25 g of Coomassie brillant blue in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid. Staining was at room temperature for 8 hours. The gels were destained electrophoretically or simply by sitting for a long period of time in destaining solution (75 ml of acetic acid, 50 ml of methanol, and 875 ml of water). Assuming an even swelling of the gels, mobility was calculated as the ratio of the distance of protein migration to the distance of dye migration. The mobilities were plotted against the known molecular weights expressed on a semi-logarithmic scale. The marker proteins used were Myoglobin (17,800 daltons), Chymotrypsinogen A (25,000 daltons), Pepsin (35,000 daltons), Ovalbumin (43,000 daltons) and Albumin (68,000 daltons). (E), Periodic acid Schiff procedure (39): Gels were stained for carbohydrate using the periodic acid Schiff procedure. The SDS was removed by the following steps; the solutions were stirred vigorously at room temperature for the following 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) 0.1% sodium arsenite,

5% acetic acid; 60 min - repeated twice; (4) acetic acid; 10-20 min. The gels were then (5) transfered 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.

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

Chromatography and Autoradiography - Thin layer chromatography was conducted in two dimensions (for polar lipids) and in one dimension (for nonpolar lipids) using Silica Gel precoated plates. Two dimensional plates were activated at 60° for 1 hour, cooled in a desiccator and developed in the first dimension with chloroform:methanol-28% (w/v) aqueous ammonia 65:35:5 (v/v/v) chromatograms were dried for about 10 min cooled in a desiccator and then developed in the second dimension with chloroform:acetone:methanol:acetic acid:water 5:2:1:1:0.5 (v/v/v/v/v)(40). Plates were obtained from two sources. Brinkman Inst. (F-254) plates gave poor results charring the chlorosulfolipids using 25% (w/v) sodium

bisulfate, 3% (v/v) H_2SO_4whereas better autoradiograms were obtained from these plates. Conversely the chlorosulfolipids were charred with facility on Supelcosil 42A (Supelco, Inc., Bellafonte, Pa.) plates although these plates were too powdery for autoradiogram preparation. These two types of plates yielded the best separations of the polar lipids. Asolectin, a mixture of lecithin, phosphatidylethanolamine and phosphatidyl inositol, was used as a chromatographic standard for these substances. It was obtained from Associated Concentrates, Woodside, N.Y. Compounds were visualized by Iodine or charring of the plate by spraying a solution of 25% (w/v) sodium bisulfate containing 3% (v/v) H_2SO_4 followed by slow heating over a hot plate. Plates were also examined under Ultraviolet radiation at 254 and 366 nm. For autoradiography, each chromatogram was sprayed with Spectrafluor (upon dilution, 4 g PPO plus 50 mg POPOP per liter of toluene) (Amersham/Searle Corp.) 3 times with 8 ml on each spray. The plates were then exposed to X-ray film, blue sensitive (code SB-54) in the dark at -16°, for a period from 1 to 10 days, depending on the amount of radioactivity spotted on the thin layer plate. Individual lipids visualized by autoradiography were scraped off the plate directly into scintillation vials. Ten ml. Bray's (41) solution was added to each vial and counted in a Nuclear Chicago Scintillation Counter Model No. 724 or a Beckman LS-250 Liquid Scintillation system.

Diphenylamine - Plates were sprayed with diphenylamine spray (Supelco, Inc.) and heated for 5 to 10 min at 100° (43). Ninhydrin - plates were sprayed with 0.2% (w/v) ninhydrin in 95 ml butanol and 5 ml 10% (v/v) acetic acid. The plates were heated at 105° for 20 min (43,45). 2,4-Dinitrophenylhydrazine- (Supelco, Inc.) was sprayed on the plate. Molybdenum trioxide - Equal volumes of solution A (40.11 g molybdenum trioxide in 1 liter 25 N H_2SO_4, boiled until the solid has dissolved) and solution B (1.78 g molybdenum powder in 500 ml solution A is boiled 15 min and cooled) are mixed and carefully diluted with 2 volumes of water. The reagent is stable for several months(43,44). Dragendorff - Solution 1, (20 ml) (1.7 g basic bismuth nitrate in 100 ml 20% (v/v) acetic acid) and solution 2, (5 ml) (40 g potassium iodide in 100 ml water) were diluted with 70 ml water before use (42,43) . Cis-Aconitate - The spray reagent of Vaskosky and Suppes (46) was used to detect quaternary amino groups. Water must be rigorously excluded for successful use of this reagent.

Phosphorus Analysis of Spots from TLC - Spots were scraped off the plates directly into test tubes and analysed by the method of Rouser (40): Concentrated hydrochloric acid (0.5 ml) was added to each tube; water and excess acid were then removed by maintaining the tubes at 170° for 30 min. Perchloric acid (0.26 ml) (70% w/v) triple distilled into Vycor) was added and the lipid digested by refluxing for 40 min at 170°. After cooling, reagents added in order were: water (0.92 ml), ammonium

molybdate (0.4 ml, 1.25% w/v), and ascorbic acid (0.4 ml, 5% w/v). Color was developed by heating for 5 min in a boiling water bath and then cooling for 10 min. The adsorbent was sedimented by brief centrifugation. The color intensity of the supernatant was determined at 797 nm in a Carl Zeiss model M4QIII spectrophotometer.

Protein Assay - The concentration of protein was determined by the method of Lowry, et al., (47) using bovine serum albumin as a standard.

The Azure A Colorimetric Assay for Sulfolipid - The procedure of Kean (48) was used: Samples were pipetted into screw cap test tubes and evaporated to less than 0.1 ml volume. To each tube was added 5.0 ml of chloroform: methanol, 1:1 (v/v), 5.0 ml of 0.05 N H_2SO_4, and 1.0 ml of Azure A solution (40 mg in 5.0 ml of 0.05 N H_2SO_4 diluted to 100 ml with water). The tubes were capped, shaken for 30 sec (Vortex) and centrifuged (300 X g for 5 min). The absorbance (Carl Zeiss M4QIII) at 645 nm of the lower phase is a molar measurement using SDS as a standard. It should be noted that the measurements of O. danica sulfolipids (with two sulfates on the molecule) must be halved to obtain molar quantities.

Sythesis of Cholesteryl Oleate - Oleic acid (278 mg) in benzene (20 ml) was cooled in an ice bath. Phosphorous trichloride (200 µl) was added and the mixture maintained in the ice bath for 1 hour. Cholesterol (389 mg) in banzene (10 ml) was added and the reaction left in the ice bath overnight. The solution was washed twice with saturated sodium bicarbonate, washed with water, separated, and the benzene dried with

anhydrous sodium sulfate. After evaporation of the solvent the cholesteryl oleate still had oleic acid which was useful in the TLC standard.

Sugar Determination in Unknown No. 1 ("Glycan") - Unknown No.1 ("peptidoglycan") was scraped off the plate, extracted with distilled water and dried to constant weight (Cahn Balance MS2T) in a vacuum desiccator (2.5 mg). The substance was then dissolved in water (1 ml). An aliquot (0.1 ml) was diluted with 1 ml of anthrone reagent (49) at 4° and thoroughly mixed. The tightly capped tubes were placed in a boiling water bath for 15 min, cooled for 10 min and the absorption compared to that of glucose treated with anthrone in like manner at 620 nm (Carl Zeiss M4QIII).

Hydrolysis of Chlorosulfolipids and Unknown No.5 - In order to quanitate the relative amounts of sulfolipid and unknown No. 5 which overlapped on the TLC plate during the [^{14}C] labelled experiment (Fig. 17,19). The mixture was hydrolyzed to produce labelled fatty acids (unknown No. 5) and labelled diols (sulfolipid) which were subsequently separated and counted. The spots were scraped off the plate into a test tube. Hydrochloric acid (1 N , 1 ml) was added and the tube placed in boiling water for 2 hours. The solution was exhaustively extracted with diethyl ether, evaporated in vacuo and chromatographed on TLC in the one dimensional solvent used for non-polar lipids.

Free Fatty Acid Composition of the Flagella Membrane - The spot identified as the free fatty acids on TLC (Fig. 20) was scraped from the plate into a test tube. The silica was exhaustively extracted with chloroform and

the combined extracts evaporated under a stream of nitrogen. The residue was heated in a boiling water bath in 2 ml methanol-boron trifluoride reagent (Applied Science). Water (2 ml) and ether (5 ml) were added to the solution. The ether was separated and combined with three subsequent ether washes. The ether solution was backwashed with water three times, dried with anhydrous sodium sulfate and evaporated in vacuo. The entire procedure was conducted under nitrogen. The fatty acid methyl esters were examined on a Perkin Elmer (801) Gas Chromatograph (flame ionization) on a six ft. column of Gas-Chrom Q (120 mesh) coated with diethylene glycol succinate (10%) and on a similar column coated with 3% OV1. The second column was used to confirm identifications. The column was programmed at 140° for 4 min then increased at 2 degrees / min to 190°. This procedure provided symmetrical peaks for analysis. The dominant error introduced into the analysis is due to the difference in detector response between polyunsaturated and the saturated fatty acid (the response is greater for saturated acids) which error can be as large as 10%.

RESULTS

Cultures - O. danica grows readily in the dark. The cultures appear yellow instead of green as they do not contain chloroplasts. After three days in sodium [1-^{14}C]acetate, 40% of the radioactivity was found in the cells, 26% appeared in the medium and 34% was gas mostly [^{14}C]CO_2. The latter was found in the NaOH traps and may contain [^{14}C]-acetate.

Isolated Flagella - Flagella preparations were examined under phase contrast light microscopy (Fig. 4) and electron microscopy (Fig. 5). They were nearly free of other cellular contamination. Isolated flagella are very delicate. In order to keep the integrity of the flagella membrane, flagella were washed with fresh culture medium. Electron microscopy showed a rippled flagella membrane (Fig. 5). This morphology may be related to some function of the flagella membrane. When sodium [1-^{14}C]acetate was added to cultures, 0.64% of the total radioactivity found in cells was incorporated into the flagella preparation. The flagella preparation represented about 4% of the dry weight of the cells.

Detachment of Flagella Membranes from Axonemes - When flagella were suspended in distilled water or TMM(10 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mM mercaptoethanol) buffer, most of the flagella membrane became released and detached from the axonemes as revealed by electron microscopy (Fig. 6). Sodium acetate buffer having the same ionic strength and pH as the medium

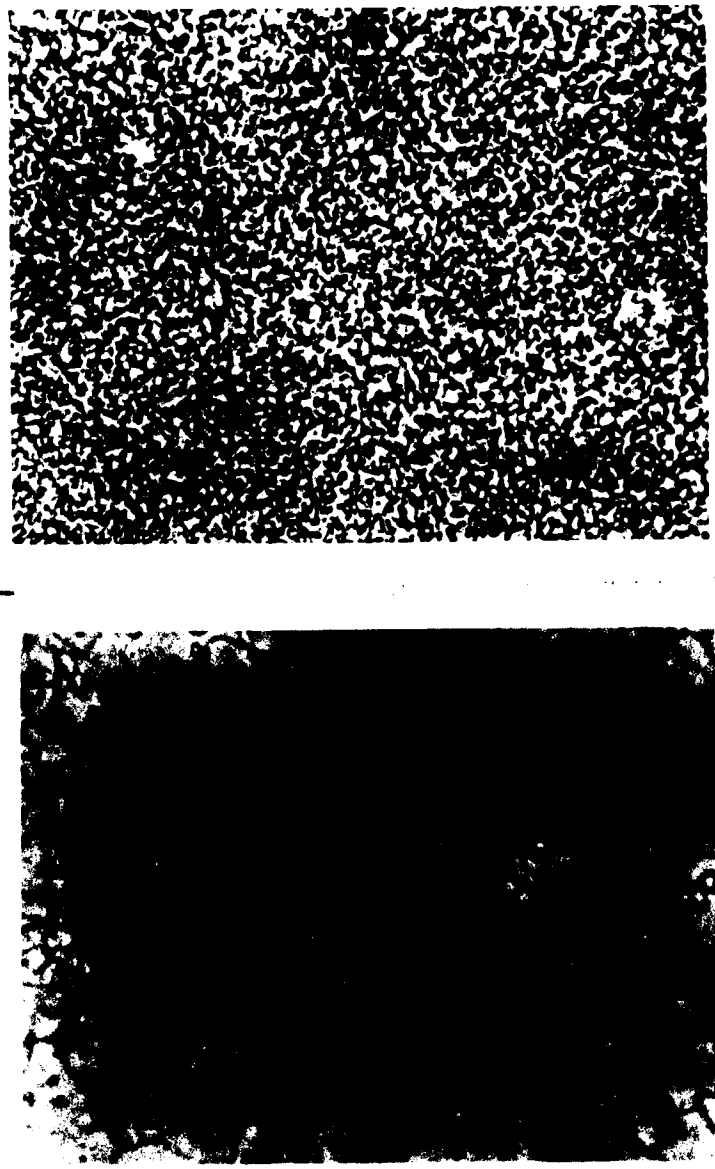


Fig. 4. Isolated flagella (0.1% Toluidine blue stained) of Ochromonas danica examined under phase contrast microscope. Upper, X 125; Lower, X 625.

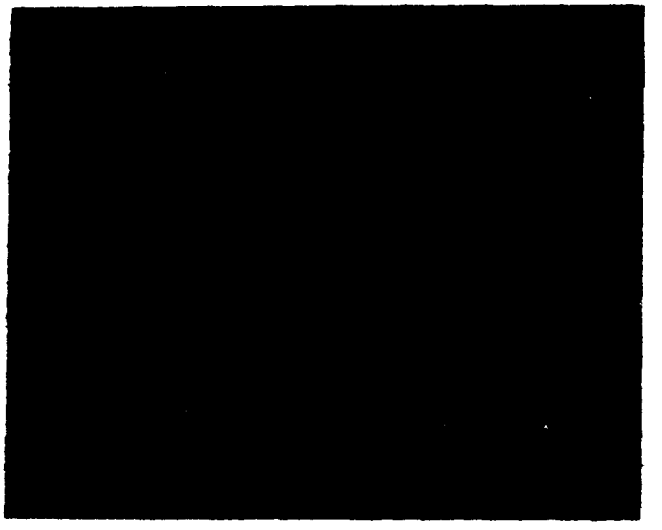


Fig. 5. Electron micrograph of isolated flagella. It shows the rippled flagella membrane. X 25,900.

Fig. 6. Electron micrograph of the flagella after suspension in TMM buffer. Most of the flagella membrane became released and detached from the axonemes. X 68,000.

also caused less although clear detachment of flagella membrane.

Appearance of Flagella Membrane - Most of the flagella membrane appeared as large membrane sheets when it was originally released from flagella as shown in Fig.6. After centrifugation at 113,700 X g for 1 hour at 4°, some membrane became small vesicles (approx. 1000 Å in diameter) as shown in Fig. 7. After centrifugation at 250,000 X g for more than 3 hours at 4°, little membrane sheets were seen under electron microscopy (Fig. 8). It seems that the centrifugation force converted some large membrane sheets into small vesicles. Some vesicles showed a flattened appearance. Flattened vesicles were converted into little membrane sheets again when the centrifugation was high enough (250,000 X g for 3 hours or more). The flagella membrane preparation appeared to be pure and showed a trilamellar structure (Fig. 7). The membrane thickness is about 80 Å . Since flagella have no internal membranes and since axonemes, mastigonemes, and extramastigoneme filaments are easily identified by their typical appearance in electron microscopy, it is safe to use this method as a criterion of flagella membrane purity. Membrane density determined by sucrose density gradient centrifugation varies slightly from 1.11 to 1.15. Repeated electron micrographic examinations of runs with the membrane in a variety of conditions (small vesicles, large vesicles, or sheets) shows variation in sedimentation in differential centrifugation relates to the form of the membrane in the preparation.

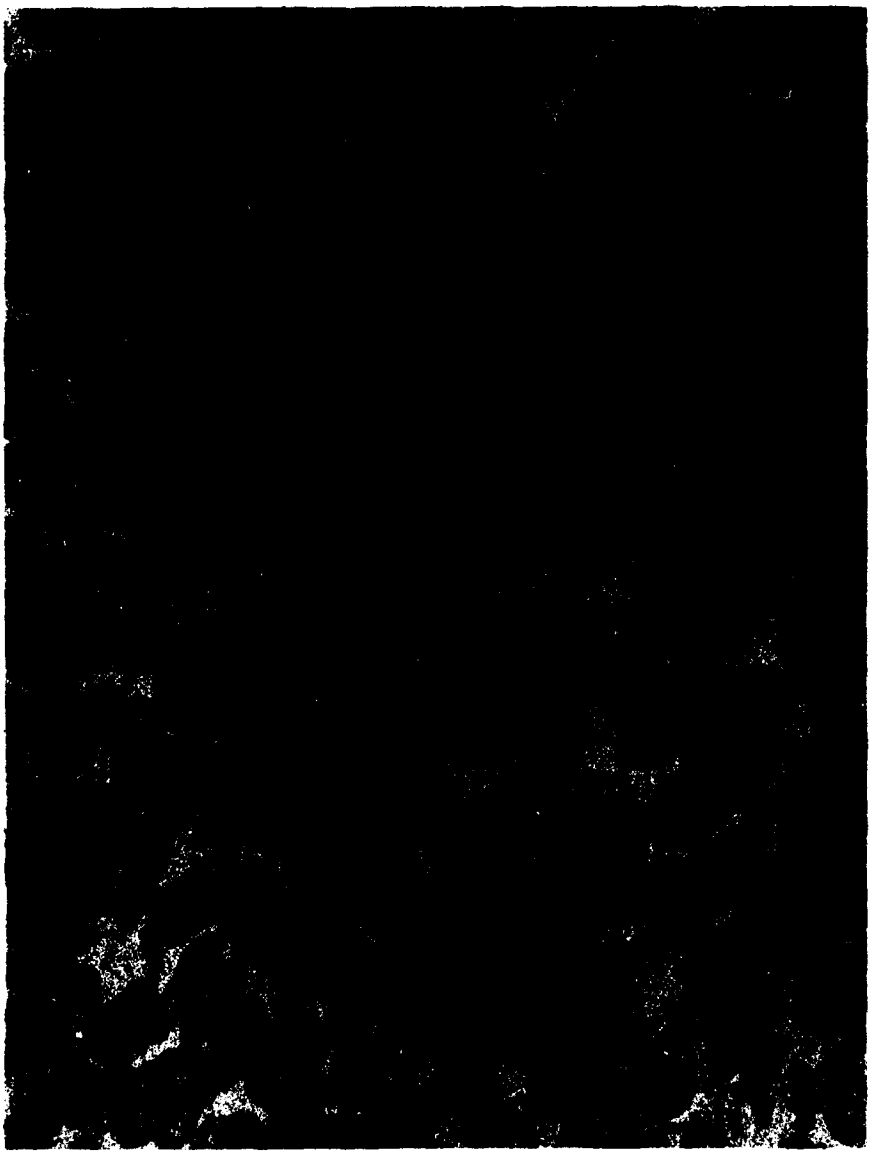


Fig. 7. Electron micrograph of flagella membrane after centrifugation at 113,700 X g. X 111,000.

Fig. 8. Electron micrograph of flagella membrane after centrifugation at 250,000 X g for 3 hours. Some membrane vesicles were converted to little membrane sheets. X 71,600.

Electron Microscopy of Other Flagellar Components - The axoneme preparation is shown in Fig. 9. There is some contamination from intact flagella membrane. Fig.11 shows a mixture of mastigonemes and membrane vesicles prior to continuous sucrose density gradient centrifugation (Scheme I). The precipitate at the bottom of the centrifuge tube is called mastigonemes Prepn. 2 and contains essentially mastigonemes with some membrane contamination. Mastigonemes Prepn. 1 consist essentially of tubular mastigonemes and filamentous cylinders (Fig.10) which could be either extramastigoneme filaments or fibrous mastigonemes since these are indistinguishable in the electron micrographs. The mastigoneme shaft is 200 Å in diameter in agreement with Bouck (17). The electron micrograph of the "extramastigoneme filaments" preparation is shown in Fig. 12. These filaments are approximately 30-35 Å in diameter and appear to vary in length. The filaments have not previously been isolated. Although we identify them as "extramastigoneme filaments" it should be understood that we could not positively identify them as such since they could be fibrous mastigonemes.

Electrophoretic Analysis of the Flagellar Components - The protein or subunits of the various flagella fractions was examined by SDS-polyacrylamide gel electrophoresis. Specific proteins could be assigned to certain flagella fractions. By comparing the protein bands and the areas of the appropriate regions of the densitometric scans of the gels, the relative purity of the various fractions was determined. Five marker proteins were used to determine the molecular weight of flagella

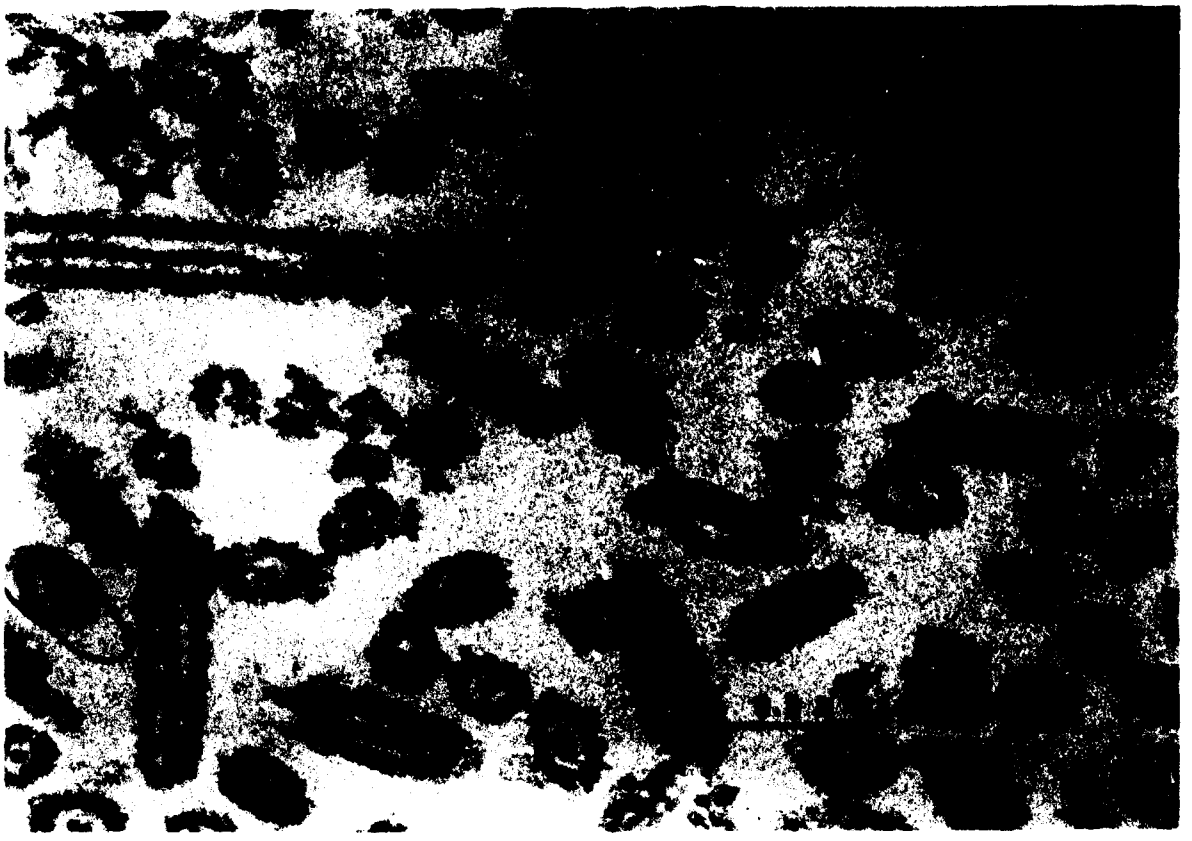


Fig. 9. Electron micrograph of axonemes. Some intact flagella and some membrane contamination were seen. X 44,700.

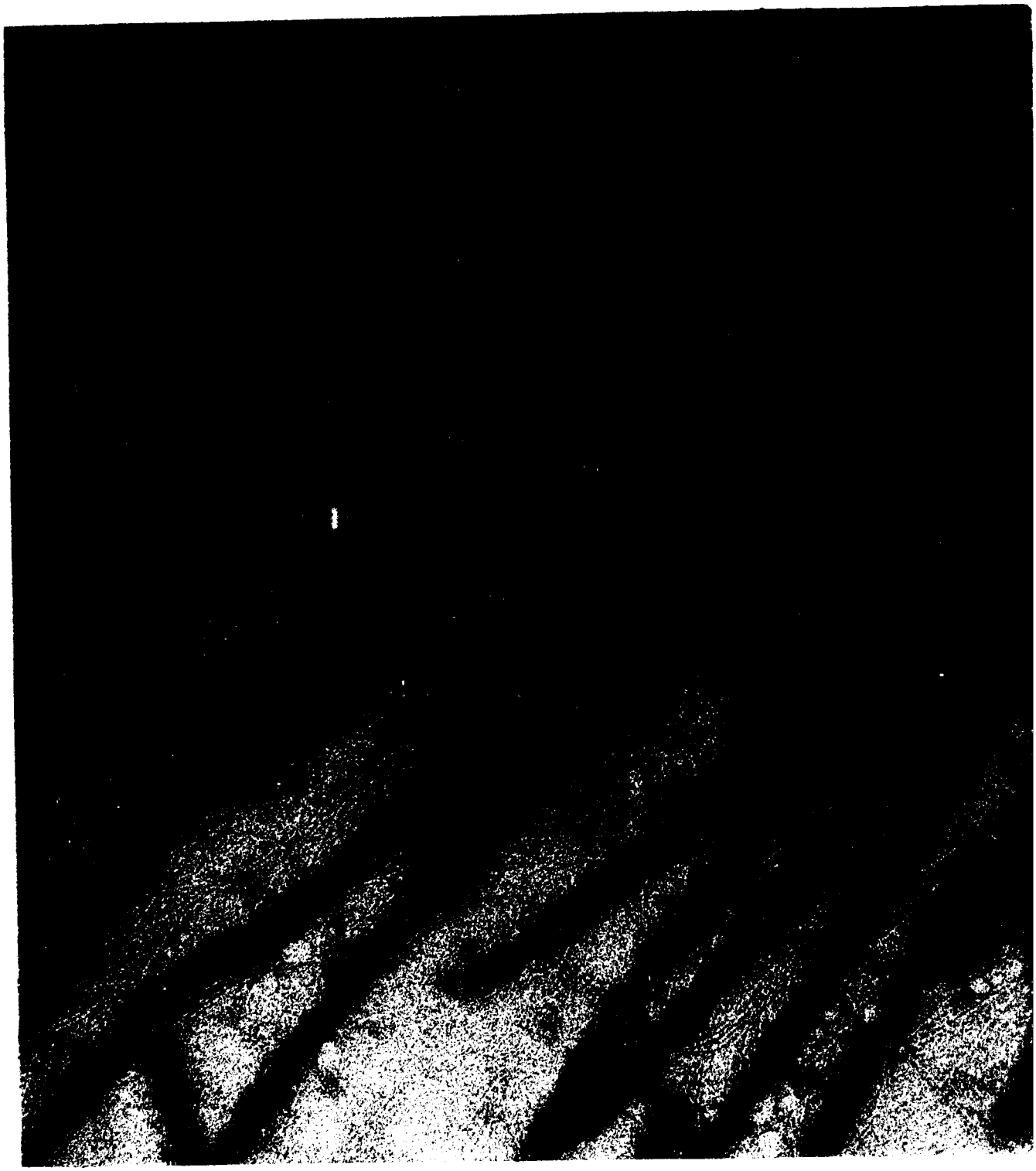


Fig. 10. Electron micrograph of negatively stained (ammonium molybdate) mastigonemes Prepn. 1. X 139,000.

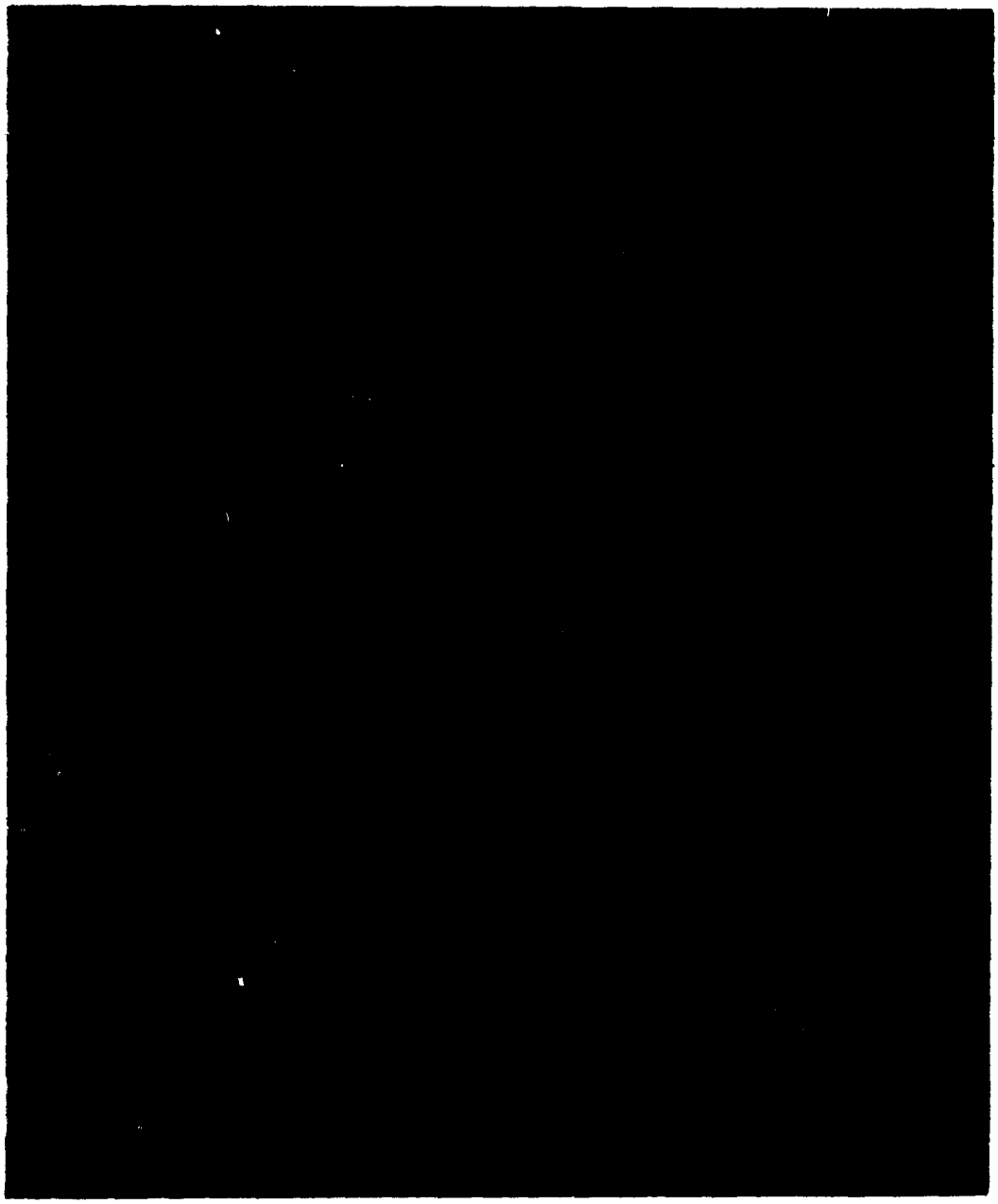


Fig. 11. Electron micrograph of a mixture of mastigonemes and membrane vesicles prior to continuous sucrous density gradient centrifugation (Scheme I) which yields mastigonemes Prepn. 2 and a pure membrane preparation. X 139,000.

Fig. 12. Electron micrograph of "extramastigoneme filaments". At present, they cannot be positively identified as such since they could be fibrous mastigonemes. X 139,000.

proteins, a plot of migration vs. molecular weight is shown in Fig. 13 .

Electrophoretic Analysis of Whole Flagella and Flagella Membrane - The whole flagella and flagella membrane protein patterns stained with Coomassie blue and the corresponding densitometric scans are shown in Fig. 14. Some protein remained on top of each gel. Any protein having a molecular weight substantially larger than 120,000 would remain at the top and was not investigated. Flagellar membrane showed five major protein or subunits having molecular weights of 54,000, 47,000, 34,500, 31,000 and 28,000 daltons and five minor protein bands.

Electrophoretic Analysis of Mastigonemes and "Extramastigoneme Filaments" - Two mastigoneme preparations were obtained. The electrophoretic analysis in Fig. 15. Shows that the mastigonemes Prepn. 1 contained 3 major protein bands. Two of them were revealed as doublets and migrated with an average velocity corresponding to 83,000 daltons, the other major protein band migrated with a velocity corresponding to 54,000 daltons. A minor protein band migrated with a velocity corresponding to 110,000 daltons. Some protein remained on top of the gel, and a heavy carbohydrate band is seen near the tracking dye. All these major and minor bands were glycoproteins since they were stained with periodic acid Schiff stain. The mastigonemes Prepn. 2 contained a major protein band which migrated with a velocity corresponding to 46,000 daltons. Additionally, this preparation contained all those proteins found in mastigonemes Prepn. 1. The electrophoretic analysis

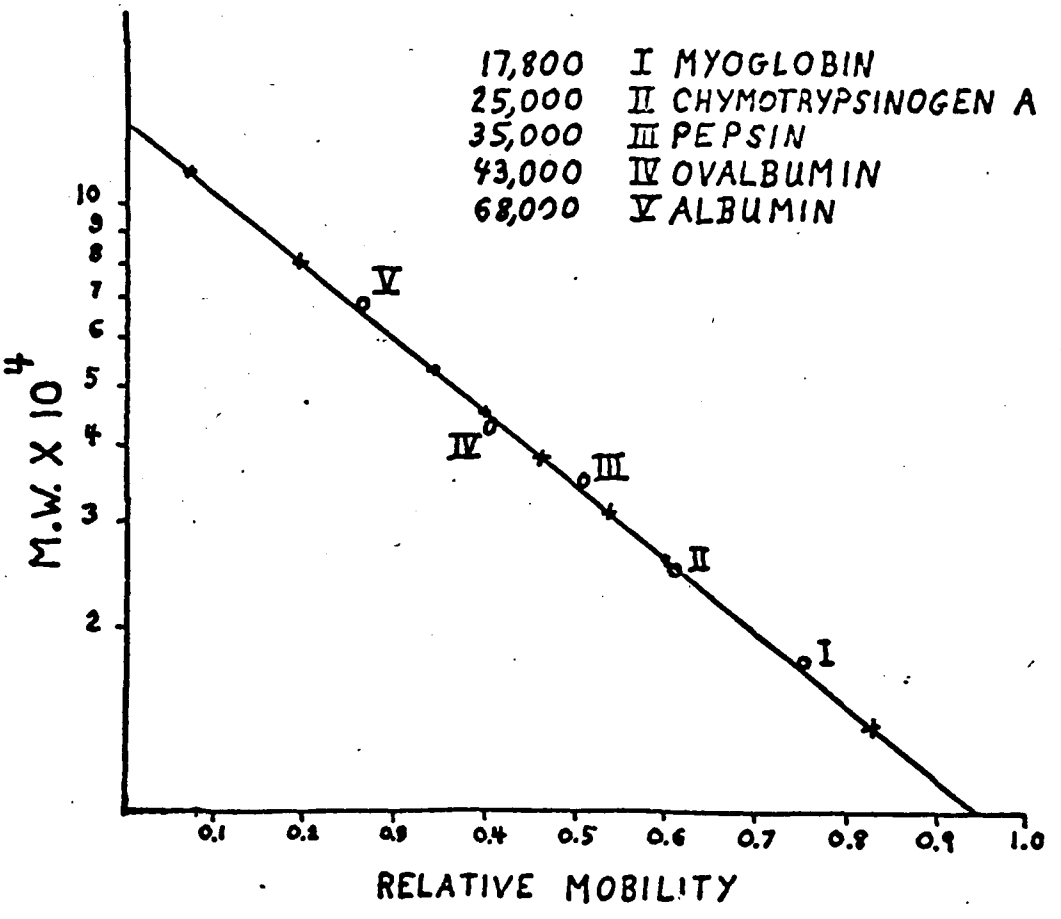


Fig. 13. Determination of the molecular weight of some O. danica flagella proteins. X = major unknown protein in a flagella fraction. o = one of the 5 marker proteins.

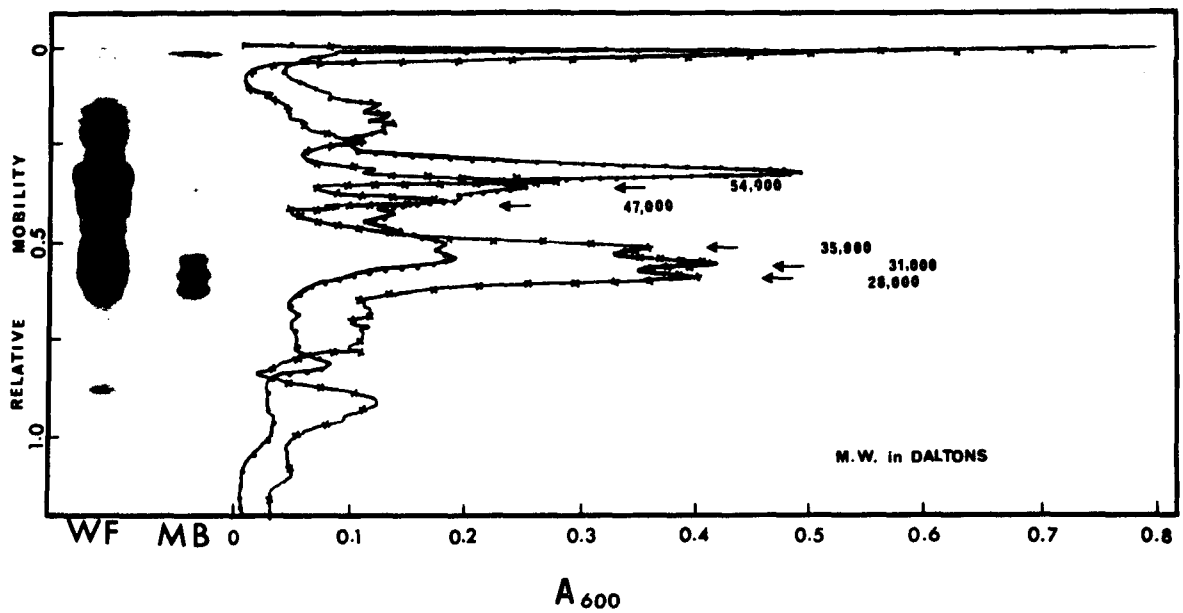


Fig. 14. Whole flagella (WF) (•—•) and purified flagellar membrane (MB) (×—×) solubilized in 1% SDS, 1% mercaptoethanol, 0.01 M sodium phosphate buffer (pH 7.0) and electrophoresed on SDS—10% acrylamide gels for 4.5 hours. Both gels were stained with Coomassie blue. The corresponding densitometric scan of the gels at 600 nm is also shown.

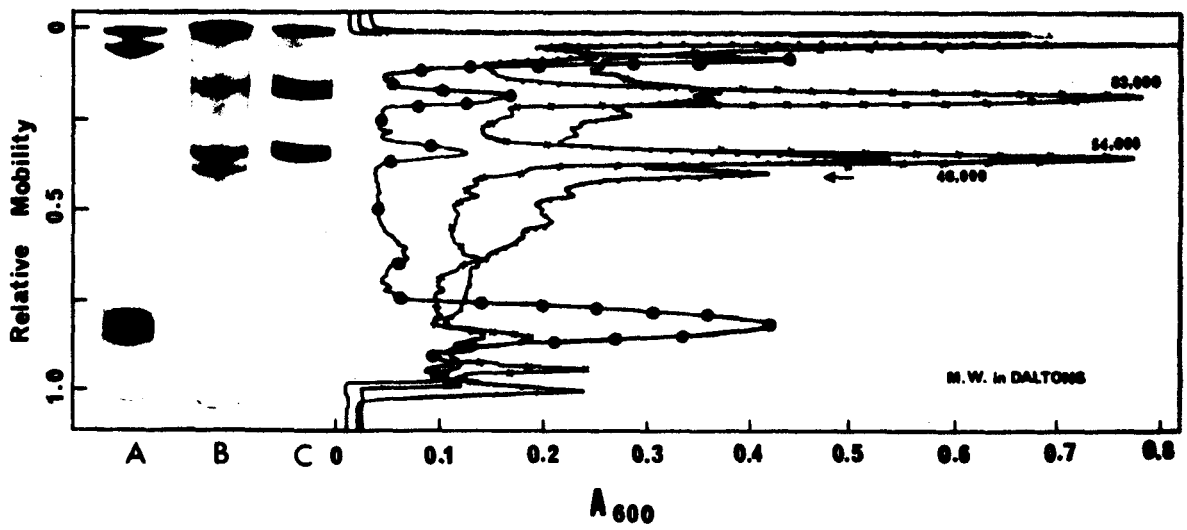


Fig. 15. Purified mastigonemes solubilized in 1% SDS, 1% mercaptoethanol, 0.01 M sodium phosphate buffer (pH 7.0) and electrophoresed on SDS - 10% acrylamide gels for 4.5 hours. The corresponding densitometric scan of the gels at 600 nm is shown. A (o—o), Mastigonemes Prepn. 1 stained with periodic acid Schiff procedure; B (•——•), Mastigonemes Prepn. 2 stained with Coomassie blue; C (×——×), Mastigonemes Prepn. 1 stained with Coomassie blue. The Prepn. numbers refer to Scheme I.

of the "extramastigoneme filaments" preparation (not shown) indicates that it contained a major protein band which migrated with a velocity corresponding to 46,000 daltons; a broad protein peak which spread over the 28-34,000 range and is probably due to membrane contamination; and a heavy carbohydrate band migrating at the same relative mobility as the carbohydrate band found in mastigoneme Prepn. 1 and Prepn. 2 (near the tracking dye). Thus the 46,000 protein band and the carbohydrate band near the tracking dye were identified with the "extramastigoneme filaments".

Electrophoretic Analysis of Axonemes - The axoneme preparation showed one major protein band having a molecular weight of about 54,100 daltons, several minor protein bands and some proteins having high molecular weights remained on top of the polyacrylamide gel (Fig. 16). It is important to note here that at least one high molecular weight protein (500,000 daltons) would be anticipated in this preparation as the dyneins (ATPases) which are the "wings" attached to each of the outer nine doublets should be present in the preparation. These proteins would be found in the origin,

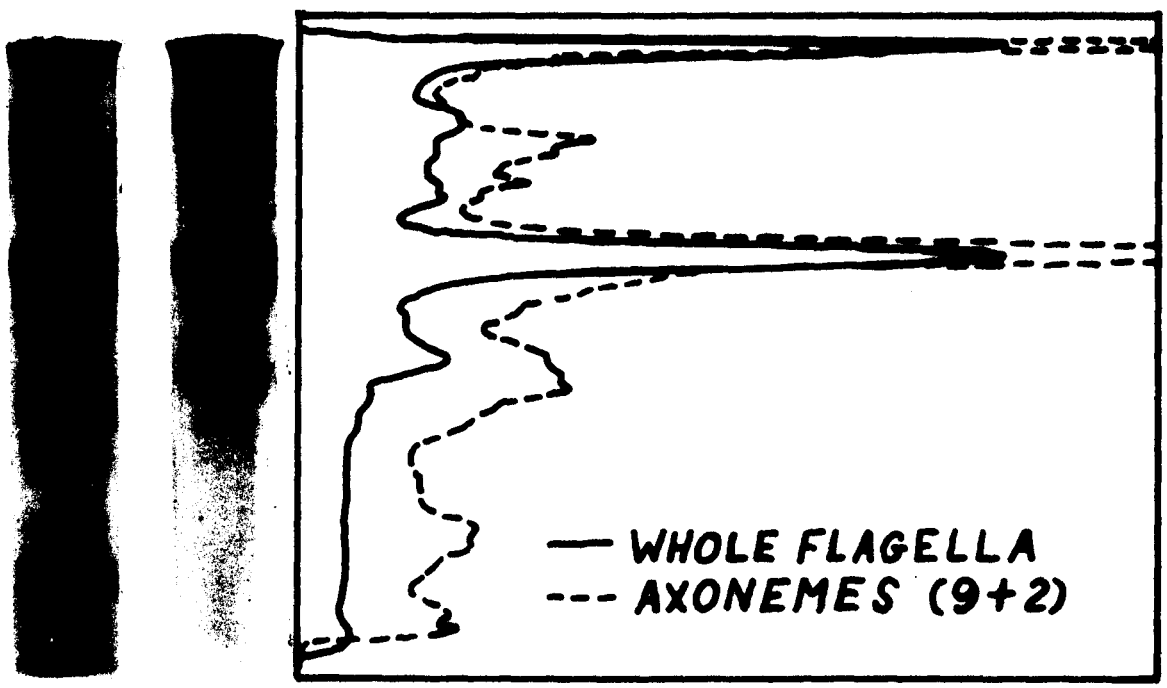


Fig. 16. Whole flagella (WF) (——) and axonemes (AX) (·····) solubilized in 1% SDS, 1% mercaptoethanol, 0.01 M sodium phosphate buffer (pH 7.0) and electrophoresed on SDS—10% acrylamide gels for 4.5 hours. Both gels were stained with Coomassie blue. The corresponding densitometric scan of gels at 600 nm is also shown.

Lipid Composition of O. danica (dark grown) - Autoradiograms of TLC separation of O. danica lipids are shown in Fig.17 and in Fig.18 . The polar lipids were separated by two dimensional TLC by Rouser's system. Phosphatidylcholine and phosphatidylethanolamine were identified by using Asolectin as a standard. The pattern obtained in this system for the polychlorosulfolipids is clearly shown. Sphingosine containing lipid had been found to be less than 0.3% of the total lipid[3], which was the limits of detection used. Nonpolar lipids migrated to the upper left hand corner of the plate. Fig.18 shows a one dimensional TLC of O. danica lipids developed in a solvent system of ether:hexane 3:7 (v/v). The nonpolar lipids were separated and the polar lipids remained at the origin. Cholesteryl oleate was synthesized and used as a standard. Stigmasterol was also used as a standard. Six sterols have been identified in light grown O. danica (50) and all these sterols migrate with the same R_f values as stigmasterol. Comparison with standards indicates that there are free fatty acid and free sterols in O. danica lipids but no sterol esters. A chromatogram with an identical pattern was obtained by spotting the whole cell directly on TLC without preextraction of the lipids with chloroform-methanol.

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

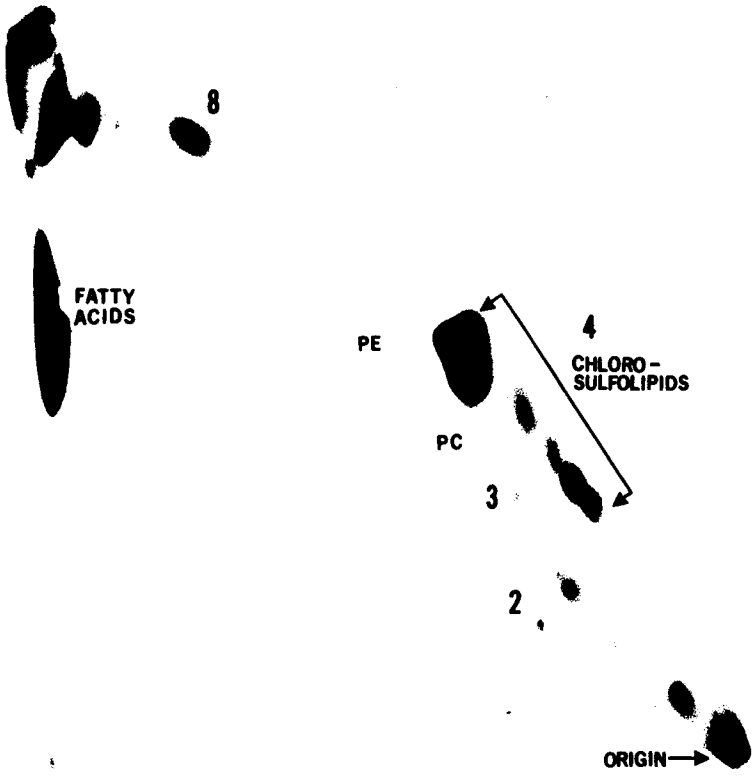

8
FATTY ACIDS
PE
4
CHLORO - SULFOLIPIDS
PC
3
2
ORIGIN

Fig. 17. Autoradiogram of a TLC of polar lipids from O. danica (dark grown). Crude lipid extract was spotted on the lower right hand corner. Blue sensitive no screen X-ray film was used. The adsorbent was Silica Gel F-254 precoated TLC plates from Brinkman Instruments Inc. Solvent system 1, chloroform:methanol:28% (w/v) aqueous ammonia 65:35:5 (v/v/v); solvent system 2, chloroform:acetone:methanol:acetic acid:water 5:2:1:1:0.5 (v/v/v/v/v). PC = phosphatidylcholine, PE = phosphatidylethanolamine.

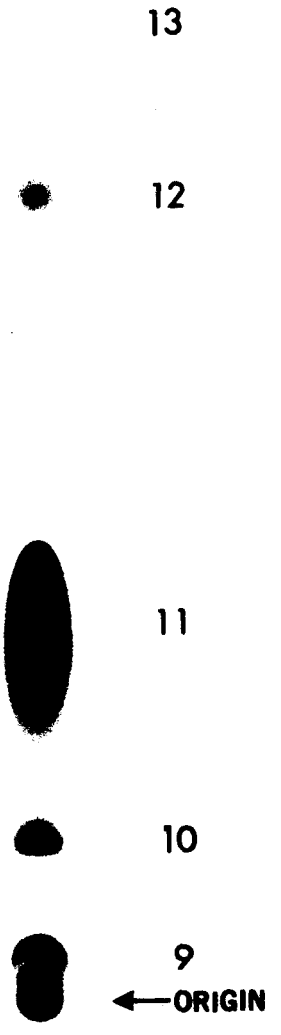


Fig. 18. Autoradiogram of a TLC of nonpolar lipids from O. danica (dark grown). Crude lipid extracts was spotted on Silica Gel F-254 adsorbent. The solvent system is ether:hexane 3:7. Blue sensitive no screen X-ray film was used.

Polar Lipid Composition of O. danica Flagella and of the Flagellar Membrane - The polar lipids of the flagella were separated by two dimensional TLC using Rouser's system. An autoradiogram of the lipids obtained from the flagella of [^{14}C]acetate labelled cells is shown in Fig.19 A. Fig.19B shows the charred TLC of the polar lipids of the flagella separated by the same solvent system. The TLC of the flagellar membrane lipid was virtually identical to that of the whole flagella. The most significant difference between the charred plate and autoradiogram is that the heavily charred Unknown No. 1 is absent in the autoradiogram. This means that Unknown No. 1 is not synthesized using acetate as a precursor. The pattern of the flagellar lipids is simpler than the polar lipid pattern of the whole cell. A further difference is the fatty acid content of the flagella which appears to be higher in the autoradiogram than it does in the charred plate.

Preliminary Characterization of Flagella Polar Lipids - A TLC of the flagella lipids cospotted with Asolectin and phosphatidic acid standard showed that the 7 polar unknowns of the flagella do not cochromatograph with phosphatidyl choline, phosphatidic acid, phosphatidyl ethanolamine, or phosphatidyl inositol. Both phosphorus analysis on each lipid and the molybdenum spray reagent for phospholipids on the chromatogram showed that the O. danica flagella membrane contains no phospholipids. Preliminary characterization of the falgella lipids (Table 1) shows that Unknown Nos. 1, 2, 3, 6, and 7 appear to be glycolipids. Unknown No. 5, and No. 8 because they react only weakly with

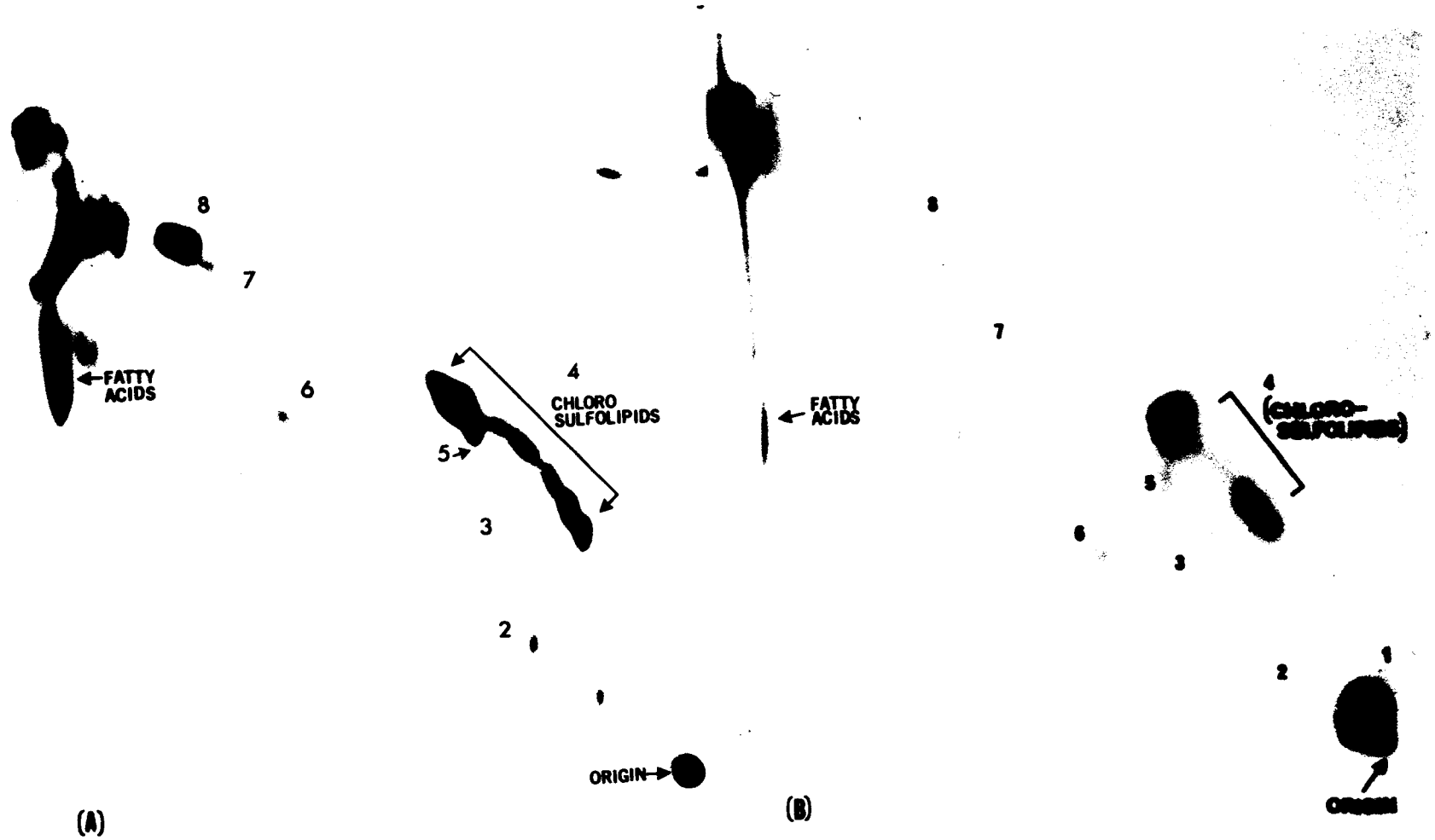

8
7
FATTY ACIDS
6
4
CHLORO SULFOLIPIDS
5
3
2
1
ORIGIN
(A)
8
7
FATTY ACIDS
(B)
4
5
3
6
2
1

Fig. 19. (A) Autoradiogram of a TLC of polar lipids from O. danica flagella. The flagella lipid extract was spotted on the lower right hand corner. Blue sensitive no screen X-ray film was used. Adsorbent: Silica Gel F-254 precoated TLC plates. Solvent system 1, chloroform: methanol:28% (w/v) aqueous ammonia 65:35:5 (v/v/v); solvent system 2, chloroform:acetone:methanol:glacial acetic acid:water, 5:2:1:1:0.5(v/v/v/v/v). (B) TLC separation of polar lipids from O. danica flagella. Adsorbent, Supelcosil 42A precoated TLC plates. Solvent, the same system as in (A). Visualized by charring with 25% (w/v) sodium bisulfate containing 3% (v/v) H_2SO_4.

TABLE I

Preliminary Characterization of the O. danica Flagella Polar Lipids

Unknown No.	Class Identification	Ninhydrin	Diphenylamine	2,4-Dinitrophenylhydrazine	Molybdenum trioxide	Dragendorff	Cisaconitate	[1-^{14}C] label
Ref.		43	43		43,44	43	46	
1	"Glycan"	+	+	+	-	+	+	-
2	Unknown	-	+	+	-	-	-	+
3	Unknown	+	+	+	-	-	-	+
4	Chlorosulfolipids	-	-	-	-	-	-	+
5	Unknown	-	+	+	-	-	-	+
6	Unknown	+	+	+	-	-	-	+
7	Unknown	+	+	+	-	-	-	+
8	Unknown	weak	weak	+	-	-	-	+
9	THAGE[a]				-		+	+

[a] Trimethyl Homoserine diAcyl Glyceryl Ether (1(3),2-diacylglyceryl-3(1)-O-4'-(N,N,N-trimethyl)-homoserine.

diphenylamine do not appear to be glycolipids. Unknown Nos. 1, 3, 6, and 7 contain nitrogen as judged by ninhydrin reaction. All unknowns reacted with acidified 2,4-dinitrophenylhydrazine suggesting the presence of aldehyde, ketone, glycoside or plasmalogen. One unknown substance, Unknown No. 1 (near the origin), appears not to be a lipid as it was not labelled with [^{14}C]acetate. It is designated here as "glycan" and will be discussed later.

The Nonpolar Lipid Composition of Flagella - The nonpolar lipids of the flagella were separated on TLC using ether:hexane 3:7. An autoradiogram of the TLC shows essentially the same pattern as the TLC visualized by charring which is shown in Fig.20 . All of the spots shown are also exposed by iodine. Spot No. 10 has the same R_f value as sterol. Spot No. 11 has the same R_f value as fatty acid. Spot 9 and spot 12 neither absorb at 254 nm nor fluoresce at 366 nm. Spot 11 and spot 13 fluoresce at 366 nm. Spot 10 absorbs at 254 nm and fluoresces at 366 nm entirely consistent with their assignment as the sterols of O. danica (50).

The "Glycan" (Unknown No. 1) - The spot near the origin (Unknown No. 1) was estimated by dry weight to be 5.8 times the quantity of sulfolipids. In order to establish this, the spot and the chlorosulfolipid spot were each scraped off the same TLC plate and extracted with distilled water and redistilled chloroform: methanol 2:1 (v/v) respectively. The chlorosulfolipids were assayed by the


SOLVENT FRONT
CHOLESTERYL OLEATE
13
12

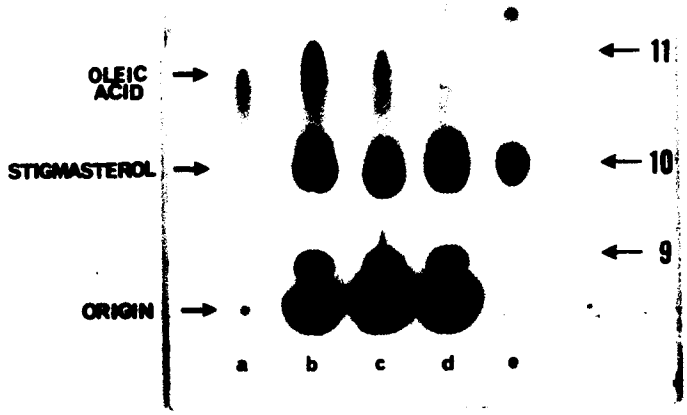

OLEIC ACID
STIGMASTEROL
ORIGIN
11
10
9
a
b
c
d

Fig. 20. TLC of nonpolar lipids of O. danica flagella visualized by charring with 25% (w/v) $NaHSO_4$ containing 3% (v/v) H_2SO_4 developed in ether:hexane 3:7 (v/v).

(a) Cholesteryl oleate and oleic acid standard.

(b) Flagella lipid extract cospotted with (a).

(c) Flagella lipid extract.

(d) Flagella lipid extract cospotted with (e).

(e) Stigmasterol standard.

Azure A method. An average molecular weight of 600 was used for the chlorosulfolipid. The unknown spot on TLC shows a strong positive reaction with Dragendorff reagent suggesting the presence of a quaternary amine group. This same unknown spot is not synthesized from [1-^{14}C]acetate as revealed by autoradiograms of thin layer chromatograms although the spot chars heavily. It shows positive reaction with diphenylamine reagent. Carbohydrate was quantitatively assayed as 35% of its weight using the anthrone reagent. The spectrum of the anthrone product shows a major peak at 425 nm and a second broad peak at 640 nm identical to glucose. The implications of the former peak are not clear. These data suggest the absence of fatty acid in this lipid substance and the presence of carbohydrate. This unknown would constitute about 62% of the total lipid based on its weight ratio to the chlorosulfolipids if it had been included in the lipid composition. Its phosphorus content is less than 0.067% by weight. It's protein (or phenolic and peptide) content is less than 4% by weight judged by the Lowry assay using bovine serum albumin (BSA) as a standard. The spot also reacted positively with ninhydrin.

Quantitative Analysis of Lipids in the Flagella and in the Cells - Sodium [1-^{14}C]acetate was added to a culture of cells in the log phase of growth and the culture maintained for 3 days. The label was used as a specific quantitative assay of the cellular lipids. The complete absence of label in the Unknown 1 spot ("Glycan") supports this

contention as 35% of this spot has been shown to be carbohydrate. The incorporation of the [1-^{14}C]acetate was further interpreted as on a molar basis and each lipid was estimated as molar percent based on the number of [1-^{14}C]acetates incorporated into its structure. Unknown lipids were averaged as having two stearate chains based on the average fatty acid composition of the cells (51), or of the flagella as measured. Using this approach, the lipid composition is described in Table II. Fig. 17. and Fig. 19 are autoradiograms of the thin layer chromatograms of lipids obtained from dark grown cells and flagella respectively. The nonpolar lipids in each plate have migrated to the upper left-hand corner of the two dimensional plate.

Unknown No. 5 did not separate cleanly from the chlorosulfolipid mixture. The mixture was scraped from the plate, extracted from the silica, hydrolysed in 1 N hydrochloric acid and rechromatographed. The fatty acids separated from the diol mixture and were counted separately to obtain the relative amounts of each. This experiment shows that Unknown No. 5 contains hydrolysable fatty acid esters - presumably diglyceride.

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

TABLE II

Analysis of Lipids in O. danica Flagella and Whole Cells

Unknown Number	Class identification	Total cell lipids		Flagella lipids		Total cell lipids		Flagellar lipids	
		dpm%	Molar%	dpm%	Molar%	Molar% Polar	Molar% Nonpolar	Molar% Polar	Molar% NonPolar
1	"Peptidoglycan"	0		0					
2	Unknown	0.3	0.2	0.5	0.3	0.7		1.1	
3	Unknown	0.3	0.2	0.2	0.1	0.7		0.4	
4	Chlorosulfolipids	13.3	13.9	17.9	20.0	47.0		72.5	
5	Unknown	0.5	0.3	0.9	0.6	1.0		2.2	
6	Unknown	0		0.4	0.3	0		1.1	
7	Unknown	0		0.3	0.2	0		0.7	
8	Unknown	1.3	0.8	5.3	3.6	2.7		13.0	
9	THAGE[a]	17.9	12.4	3.4	2.5	42.0		9.1	
10	Sterols	14.7	14.1	28.3	28.9		20		39.9
11	Free fatty acids	39.0	49.9	30.9	42.1		71		58.2
12	Unknown	7.8	5.0	1.3	0.9		7.1		1.2
13	Unknown	1.5	1.0	0.7	0.5		1.4		0.7
	PC + PE	0.3	0.2	0		0.7			
	Unidentified polar lipids in whole cells	3.0	2.0	0		7.0			

TABLE II (Cont.)

[^{14}C]-Acetate was incubated with the cells for 3 days (8 cell divisions) in a chemically defined medium (see methods) containing amino acids and glucose. Lipids were separated on TLC and counted in a scintillation counter after being scraped off the plate. The radioactivity was considered to be only in the fatty acid portion of each lipid molecule. Molar % refers to the fraction of that lipid present based upon a ratio of acetate precursors used in the biosynthesis. The following numbers were used in this ratio for each lipid indicated: All unknowns:36 (two C_{18} chains); chlorosulfolipids:22; trimethyl homoserine diglyceride:33.3; sterols:24; free fatty acids:18. Unknown 1, the "peptidoglycan," which is 30% carbohydrate (anthrone) contained no label from [1-^{14}C]-acetate. It was estimated by its weight in mg to be 5.8 times the weight of the chlorosulfolipids.

[a]THAGE = Trimethyl Homoserine diAcyl Glyceryl Ether.

TABLE III

Free Fatty Acids of O. danica Flagellar

Chain Length	Double Bonds					
	0	1	2	3	4	5
14	13.5					
16	14.9	4.7				
18	8.8	15.8	12.5	1.7	2.4	
20	1.2			4.5	1.6	
22	Tr				4.9	1.5

Values are expressed as % composition based on a gas chromatogram of the methyl esters on diethylene glycol succinate (10%) on Gas-Chrom Q. The unsaturated esters include: palmitoleate ($C_{16:1}$), oleate ($C_{18:1}$), lineoleate ($C_{18:2}$), γ-linolenate ($C_{18:3}$), and arachidonate ($C_{20:4}$). Traces (Tr) of α-linolenate and thirteen other esters was observed. Since a flame ionization detector was used the polyunsaturated acids are present in relatively higher amounts and the saturated fatty acids in relatively lower amounts than indicated.

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

TABLE IV

Lipid to Protein Ratio

The lipid analysis are based on radioassay of the lipid extract of cells cultured in the presence of [1-^{14}C]acetate. The specific activity of the isolated lipids was found to be 8.0 X 10^5 dpm per mg lipid. It was assumed that the specific activity was the same in each fraction although the determination of the specific activity was made on the whole cell lipid extract. The protein was estimated with the Lowry method (47) using bovine serum albumin as a standard.

Fractions	Lipid/Protein (w/w)
Whole Cells (dark grown)	1.1
Flagella	0.46
Flagella Membrane	1.3

DISCUSSION

Culture - O. danica does not develope chloroplast when cultured in the dark. Chloroplast represents a substantial fraction of cell weight in light grown cells. The chloroplast is fragile and will cause heavy contamination of other fractions upon cell rupture. Dark grown cells provide more appropriate cellular material for flagella isolation. Dark grown cells also provide a less complicated system for comparison of lipid and protein compositions of the whole cell with those of the flagella.

Isolation of Flagella Components - A simple and mild isolation procedure has been developed which yields a pure flagella membrane preparation, an axonemes preparation, pure mastigonemes and an "extramastigoneme filaments" preparation. O. danica is deflagellated by means of a vortex mixer at 4°. This is the mildest deflagellation method yet described. O. danica mastigonemes had earlier been isolated after treatment with Sarkosyl (17). Chlamydomonas flagellar mastigonemes, membrane and axonemes were isolated after addition of a nonionic detergent such as Triton X-100, Nonidet P-40 or Sarkosyl (19). The use of detergent to isolate the membrane may well have affected the quality of the membrane preparation. Cilia axonemes were isolated after digitonin treatment (21, 52). In the isolation procedure herein described the membrane was obtained without the introduction of any detergent. All fractions of the flagella including the "extramastigoneme filaments" were recovered.

Flagella Membrane Protein - O. danica flagella membrane consists of five major proteins (Fig. 14) having molecular weights of 54,000, 47,000, 34,500, 31,000 and 28,000 daltons (Fig. 13) and may contain some proteins larger than 120,000 remained on top of SDS-acrylamide gel. When Chlamydomonas flagella membranes were analyzed by electrophoresis on SDS-urea acrylamide gels, one major protein band was observed. It had a molecular weight considerably greater than 170,000 daltons (19). The electrophoretic conditions (especially the percent acrylamide used in the gel) were sufficiently different to allow a closer examination of a different molecular weight range. The presence of a large protein band at the origin of our gels is consistent with the Chlamydomonas result. Those experiments however may not have picked up the proteins we identified. The protein at 47,000 daltons is in the actin range of molecular weights.

Mastigonemes - The SDS-acrylamide gel electrophoretic analysis of mastigonemes Prepn. 1 displayed 3 major proteins. The analysis of mastigonemes Prepn. 2 displayed an additional major protein band migrated with a velocity corresponding to 46,000 daltons (see the section on Results). A major difference between mastigoneme Prepn. 1 and Prepn. 2 can be seen from the electrophoretic pattern to be the presence of a large amount of "extramastigoneme filaments" in Prepn. 2 whereas Prepn. 1 lack these filament proteins. Additionally, the proportions of the protein components at 54,000 and at 83,000 in Prepn. 2 and in Prepn. 1 differ suggesting that a further separation was achieved.

The isolated mastigoneme fraction presumably contains a mixture of fibrous and tubular mastigonemes. The fibrous mastigonemes presumably are constituted of different protein(s). O. danica mastigonemes contain a tubular shaft, and three types of extramastigoneme filaments of different lengths (17,18). Additionally, the fibrous mastigonemes are indistinguishable in electron micrographs from the extramastigoneme filaments. The band at 83,000 daltons is probably due to fibrous mastigoneme protein. Since all mastigoneme proteins are glycoproteins, the molecular weight should be accepted only tentatively because some glycoproteins do not migrate in SDS-acrylamide gels at rates proportional to their true molecular weights (53). Additionally, the sensitivity of the periodic acid Schiff stain is much lower than that of Coomassie blue. Mastigoneme glycoproteins, however, gave strong reactions with the periodic acid Schiff stain.

Tubular mastigonemes have been previously isolated by Bouck (17), solublized in urea, Tris EDTA, and electrophoresed on urea-containing 7.5% acrylamide gels, yielding four Coomassie blue stained bands. All these bands stained with periodic acid Schiff as well. Because he did not use SDS-polyacrylamide electrophoresis, it is hard to compare his results with the SDS-polyacrylamide gel pattern found in this laboratory. It is clear that there are at least four mastigoneme glycoproteins. Chlamydomonas mastigonemes contain a single glycoprotein of about 170,000 daltons (19). This organism, however, has a single type mastigoneme without extramastigoneme filaments.

In nonregenerating cells, presumptive mastigoneme-like structures, lacking lateral filaments, were found within the perinuclear continum. However in the regenerating cells these structures could also be localized within the cisternal of the Golgi complex and within vesicles free in the cytoplasm. There they possessed all the morphological characteristics of the mature mastigoneme, including extramastigoneme filaments. The flagellum itself possesses two rows of mastigonemes from the first appearance of the flagella stub, which indicates that mastigonemes are attached concomitantly with flagella growth in O. danica (17). The extramastigoneme filaments are added to the mastigoneme shaft in the Golgi complex. It was postulated by Bouck that these extramastigoneme filaments are wholly or in part carbohydrate (17). The extramastigoneme filaments isolated in this laboratory showed that it does indeed contain a large quantity of carbohydrate.

Although no one knows what the physiological function of the glycoprotein is, it seems that it could serve two different functions that are not mutually exclusive. The first is that of recognition; especially in gamete recognition and agglutination which serves to allow the cells to recognize one another. The other possible function is that it serves as a very primitive form of cell wall giving more rigidity (10). In red blood cells, carbohydrate portions of both glycoproteins and glycolipids are fixed in orientation on the external membrane surface (54,55). Neither the nature nor the relationship of the carbohydrate to the mastigoneme protein is yet known.

Axonemes - Axonemes were isolated using these procedures with a small amount of intact flagella contamination. Evidence for this statement is derived from the identification of some labelled lipids in this fraction during the [^{14}C]acetate labelling experiment. Additionally, intact flagella contamination was observed in the electron micrographs of axonemes, although the SDS-acrylamide gels did not show membrane pattern in the axoneme preparation. The gel showed one major protein band at about 54,100 daltons. These results should be compared to those of Witman, et al. (19), who studied Chlamydomonas axonemes and to Olmstead, et al. (56), who compared the microtubules of neuroblastoma cells, brain and Chlamydomonas. These investigators found two tubulin proteins dominated the SDS-acrylamide gel pattern. The proteins had molecular weights of 53,000 and 56,000 daltons. The identification of a single tubulin band in O. danica may be due to two tubulins having the same molecular weight, or the occurrence of only one tubulin in O. danica flagella. Judging from the gels, however, it is just conceivable that two tubulins might overlap, although repeated runs never showed a double band.

The Flagellar Membrane Composition - The membrane preparation from the flagella of O. danica has three major constituents: protein, lipid and what appears to be a "glycan". The lipid to protein ratio (Table IV) is 1.3 and the lipid to "peptidoglycan" ratio is 0.6. Nearly all of the lipids of the membrane appear to be unique. A prominent exception is the sterols, which appear to be typical algal sterols (50). There are no phospholipids in the membrane. The dominant lipids, in addition to the sterols appear to be free fatty acids and a mixture of chlorosulfolipids derivatives of 1,14-docosanediol-1,14-disulfate(32,33) and a mixture of chloro-substituted derivatives with up to six chloro groups replacing hydrogens on the chain (29,30,34,57,58). The fatty acids could conceivably be artifacts produced in extraction, etc. although every attempt was made to minimize this possibility. The chlorosulfolipids, however, cannot be considered an artifact. The identification of these substances as major membrane components is of significance since they have charged polar groups essentially at both ends of the molecule and the molecule is too short to traverse the membrane or to reside as an important contributor to the bilipid leaflet if both polar groups are at the surface.

Quantitative Analysis of the Lipid Composition - The lipid composition can be analysed quantitatively by dry weight, by densitometric scan of charred TLC plates or by radioactivity using [^{14}C]labelled precursors of lipids. When unknown lipids are present the dry weight does not give

the molar ratio of the lipids. Quantitative assay by charring is tedious, requires specially prepared plates and requires standard knowns for each spot to account for the variable charring response which is characteristic of each compound.

Radioactive labelling with [1-^{14}C]acetate would give a molar response provided that it did not label the polar group of unknown diacyldiglycerides and that the fatty acid composition of the sample was known. Additionally the sterols and other nonpolar lipids are labelled in predictable ways depending upon the structure of the lipid.

The use of this approach for this system is based upon the composition of the chemically defined culture medium which includes several amino acids and a large supply of glucose. It was felt that the carbohydrate and protein of the cells would be poorly labelled from [1-^{14}C]acetate at best. This was confirmed experimentally by the absence of radioactivity in the Unknown 1 which contains 35% carbohydrate.

This method appears therefore to be useful and relatively simple for establishing the molar ratios of the lipids. In order to calculate these ratios some assumptions are made with respect to the structures of the unknowns. An analysis of the fatty acids of O. danica shows an average chain length of just under eighteen carbons. All six unknowns, which amount altogether to 9% of the total lipids based on raw counts, were considered to be diacyldiglycerides. The chlorosulfolipids were considered to have eleven acetates incorporated into each molecule (59,60) although some tetracosane is undoubtedly present. The sterols are

considered to have twelve carboxyl carbons incorporated into the molecule based upon their structures (57) and the general biosyntheric route of sterols. The alkyl side chains of the plant sterols are derived from methionine (61).

Some error is introduced by these approximations but the error is calculated to be less than 3% for any case and the experimental error is greater than that for such variations as growth conditions and variations in nutrition.

Polar Lipid Composition of the Flagellar Membrane - The lipid composition of the flagellar membrane and that of whole flagella were found to be identical by two dimensional TLC. The results reported hereafter are based on analysis of whole flagella. The chromatograms are shown in Fig. 19. The list of polar lipid components on those chromatograms can be found in Table II. Table I shows their reactions with a variety of specific reagents.

Probably the most outstanding facts about the membrane polar lipid composition is the total absence of phospholipids and the presence of a dominant fraction (72.5 molar percent of polar lipids) of chlorosulfolipids. The former, although surprising, is not as surprising as the latter. The absence of phospholipids does not have important implications to the membrane structure as they may be replaced by other ionic or polar lipids with analogous structure. This has already been noted in O. danica by Elovson who has identified 1(3),2-diacylglycerol-3(1)-O-4'-(N,N,N-trimethyl) homoserine as one of the major lipids in the phytoflagellate with steric and ionic-hydrophobic structural properties

similar to those of the phospholipids (62). One may presume that Unknowns 2, 3, and 5-9 are analogous in their steric and ionic-hydrophobic structures to the phospholipids. These compounds, however, constitute together only 27.5 moles percent of the total polar lipids in the flagellar membrane. This is a maximum since the structures of these compounds are Unknown. The diacyl-homoserine glyceryl ether only accounts for less than 10% of the polar lipids in the flagella membrane although it constitutes 42% of the polar lipids in the whole cell. To our knowledge this is the first report of a membrane totally devoid of phospholipids. This fact has a significance of its own since the phospholipids are generally considered critical components of biological membranes (10,12,63,64,65).

Another aspect of the analysis of the flagellar membrane polar lipids that emerges as an important finding is the presence of a dominant fraction of chlorosulfolipids. These lipids are unsuitable for forming bilayer membranes as they are (1) very water soluble; (2) would have a second ionic group too deep in the hydrophobic region of the bilayer for stability; and (3) could not "loop" back so as to have both groups on the polar surface of the bilayer as the chain would only penetrate about eight carbons into the hydrophobic layer. These factors make a bilayer of the chlorosulfolipids possible only if there are positive charges deep in the hydrophobic region to shield the negative sulfate groups. (Sulfate must be charged at any physiological pH since the pK is below 1).

Only two such cations come to mind - one is divalent metal ions which would bridge two such groups and the other is hydrophobic protein or other macromolecules which penetrate the bilayer. It is possible to distinguish between these and studies are planned to make this distinction.

Several laboratories have noted the contiguity of flagellar or cilia membranes and the cell membrane. The electron micrographs obtained by us are in complete agreement with this observation. Additionally, R.B. James and T.H. Haines have obtained freeze fractures of O. danica cell membrane (66) which show the membrane has a fracture plane typical of bilayers. It would appear, therefore, that the awkward structure of the chlorosulfolipids is somehow compensated in the bilayer by other membrane components. These data exclude the suggestion made by Brown and Elovson (62) that the diacylglycerol trimethyl homoserine ether, "rather than the detergent-like chlorosulfolipids, largely substitutes for the usual phospholipids in membrane bilayer structures in O. danica".

Of the unknown polar lipids Unknown 8 dominates the flagella membrane composition. This is the only unknown polar lipid that does not react with diphenylamine. The others are apparently glycolipids. This suggests a variety of unusual glycolipids in O. danica. It should be noted that O. danica could only contain less than 0.3% of its polar lipids as sphingosine[3]. Since that was the limits of detection used in an analysis of the long chain bases.

Nonpolar Lipid Composition of the Flagellar Membrane - The nonpolar lipids of O. danica flagellar membrane have four major components. The bulk of the nonpolar lipids consists of the sterols and free fatty acids. The latter will be discussed in the next section. Two unknown classes (Unknown 12 and 13) constitute less than 2 molar percent of the nonpolar lipids. They are not sterol esters as they do not cochromatograph with cholesteryl oleate. The sterols were previously characterized in this laboratory as ergosterol, brassicasterol, 22-dihydrobrassicasterol, clionasterol, poriferasterol and 7-dehydroporiferasterol (50). The relative composition of the sterols are approximately that reported for the whole cell. It is interesting to note that the molar percent of sterol are in excess of that for all of the polar lipids taken together-including the chlorosulfolipids. This is in agreement with many reports of widely variant polar lipid to sterol ratios (67).

Free Fatty Acids - Free fatty acids represent 42 molar % of the total lipids in the flagellar membrane. This surprisingly high quantity of free fatty acids can be explained in three ways. It could be present in the flagellar membrane or be produced by a very active lipase in the lipid extract or result from a labile hydrolysis of a "glycan". O. danica grows in acidic media from pH 4 to pH 5. Cells do not grow well in media at pH's higher than 5. Free fatty acids become charged at pH's higher than 4.5, which is, interestingly enough, coincidental with the pH limit of growth of O. danica. It is conceivable although unlikely that the bulk of uncharged free fatty acids fit in the membrane in the

acidic pH range (pH 4-5) and this may offer one explanation to the inability of O. danica to grow at neutral or higher pH which is optimal for many other organisms. On the other hand, active phospholipase and galactosidases of the photosynthetic tissues of plants have been reported in lipid extracts and the addition of boiling isopropanol has been employed to inactivate the enzymes prior to extraction by chloroform: methanol 1:1 (v/v), which appears to activate the enzymes (68). It is also conceivable that for defensive or nutritive purposes, O. danica has a very high lipase activity. If this were so, the addition of chloroform-methanol would result in enzymatic hydrolysis of the lipids allowing the accumulation of a large quantity of free fatty acids. An attempt to avoid the lipase problem was made by spotting the water suspension of whole flagella and whole cells on TLC plates respectively before treatment with any organic solvent. Under these circumstances it is presumed that the lipase would not have an opportunity to attack lipids. Furthermore the lipase would be denatured when the spot dried under nitrogen prior to development. The developing solvent serves as an extracting solvent as well. This method is especially applicable when highly radioactive material is available as only small amount of material is extracted by the developing solvent and a highly sensitive visualizing method is available. The autoradiograms showed that the relative quantity of free fatty acid is at least as much using this procedure as it is with lipid extracts. This experiment appears to

exclude lipase activity as an explanation for the large quantity of free fatty acids. A third possibility is that the free fatty acids are derived from the "glycan" in a labile linkage. This seems to us most likely and is discussed in the next section.

"Glycan (Unknown No. 1) - Fig. 19B shows that near the origin, there is a heavily charred spot, not completely separated from the origin. This spot is seen only in the flagella lipid extracts developed in two dimentional TLC and visualized by charring. An autoradiogran (Fig.19A) shows that the unknown is not labelled by [1-^{14}C]acetate indicating the absence of fatty acid. The boundary of the unknown spot and origin is easily seen by its color and pattern after exposure to various spray reagents.Dragendorff reagent reacted with the spot and gave an orange color while the origin remained white. The same result was obtained with 2,4-dinitrophenylhydrazine. Diphenylamine and ninhydrin reacted strongly with both the origin and the unknown but differently and the boundary between them was shown clearly. Unknown No. 1 gave a positive reaction with diphenylamine, 2,4-dinitrophenylhydrazine, ninhydrin and Dragendorff reagent suggesting the presence of carbohydrate, aldehyde, ketone or glycoside, amine, amino acids or amino sugars and quarternary amino group. Quantitative anthrone analysis of the spot isolated from two dimensional TLC plates showed it contained 35% carbohydrate. The spectrum of the anthrone product showed only hexose and not pentose or deoxysugars were present (69,70). This unknown is most likely a "glycan". Conceivably the spot is a mixture of the polar groups accumulated after hydrolysis of polar lipids by lipase. The latter is an

attractive explanation of the Unknown No. 1 as it also explains the large amount of free fatty acids (42 molar percent) which appear in the flagellar lipids. As was indicated in the discussion on free fatty acids in the flagellar membrane preparation the spotting of wet flagella directly on the TLC plate did not alter the relative amount of the fatty acids. In addition the molecular weight of the polar group of a diacyl lipid derived from such lipase activity would have to be greater than 1600 to explain the relative amounts of the free fatty acids and the unknown spot. The expression "glycan" is therefore used to describe the substance although its molecular weight is not known - nor is it known that a pure compound is even present at this spot. There is a possibility, furthermore, that the "glycan" and the fatty acids are related if one considers that fatty acid esters with such a polymeric substances are particularly labile due to the structure of the "glycan". Such an explanation is consistant with the thin layer chromatography and also with the molecular weight ratios.

The association of actin with the isolated plasma membrane of Acanthamoeba castellanii revealed from electron micrographs has been reported (71). The actin free plasma membrane consists of 37% protein, 31% lipophosphonoglycan, a macromolecule that contains neutral sugars (26%), amino-sugars (3.3%), aminophosphonates (10%), phosphate (3.2%) and esterified fatty acids (14%) (72). An interesting comparison of the Acanthamoeba lipophosphonoglycan to this unknown includes the carbohydrate content (35%) and the free fatty acids if included as part of the unknown (15%). The differences include the absence of phosphorous in the

Ochromonas danica unknown and the presence of considerable quarternary amine.

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