THE SUGAR COMPOSITION OF THE FLAGELLA OF *OCHROMONAS DANICA*

-by-

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

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A B S T R A C T

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The amino and neutral sugar content of the lipids, membrane and mastigonemes of the flagella of Ochromonas danica were characterized. The hexosamine content was determined colorimetrically. Glucosamine was identified as the only amino sugar present with an amino acid analyzer. The neutral sugars were characterized as the trimethylsilyl ethers of methyl glycosides on gas liquid chromatography and as methyl glycosides on thin layer chromatograms. The lipids contained 95% glycerol, 2% galactose and 3% inositol. No other sugars were detected. The neutral sugars found in the membrane, mastigonemes and whole flagella were rhamnose, fucose, xylose, mannose, galactose and glucose. Uronic acids were absent. The major sugar in all three fractions was rhamnose. A small amount of glycerol was found in each fraction. The molar percent of each sugar in the whole flagella was similar to that in the mastigonemes. The weight percents of neutral sugars in the membrane and in the mastigonemes was 1 and 17 respectively. The membrane contained glucosamine (0.5%), rhamnose (0.35%), fucose (0.22%), xylose (0.09%), galactose (0.22%) and glucose (0.06%).

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INTRODUCTION

Membrane-bound glycolipids and glycoproteins are only now being extensively studied. Their chemical compositions and structures are being elucidated with newly available analytical techniques and their biochemical activities are beginning to be understood on a molecular basis. An understanding of the structures and activities of the compounds also gives insight into membrane biochemistry and biosynthesis.

The polypeptides of a membrane fall into two categories based on the ease with which they are extracted from the membrane. Peripheral or extrinsic proteins can be extracted by simple manipulations, such as changes in the ionic strength or the pH of the suspending medium. Integral membrane proteins are released in significant amounts from the membrane only when detergents or other chaotropic agents are used (1).

Integral proteins have an α-helical content of about 40%; there is little β-structure. The low amount of β-structure indicates that the protein is not predominantly spread out as a monolayer on the lipid surface. Rather, there is extensive hydrogen-bonding in the portion(s) that faces water and the peptide sequence that is buried in the bilayer. Freeze cleavage indicates that there are more protein aggregates on the inner half of the membrane (proteins that do not transverse the bilayer) than on the exterior half of the membrane (proteins that transverse the bilayer) (2).

There is extensive evidence that the preponderance of proteins that transverse the bilayer are asymmetrically oriented in the membrane, with the N-terminal portion of their chain and the protein's prosthetic group, such as oligosaccharides or hemes, facing the extracytoplasmic space and their C-terminal in the cytoplasm. The sidedness of glycoproteins on the membrane has been extensively studied.

Ferritin derivatives of lectins, proteins that bind specific sugars, react with the outside surface of plasma membranes and the extracytoplasmic surface of intracellular membranes (3). Nueraminidase removes all of the sialic acid of erythrocytes (4) and galactose oxidase followed by borotritide labels galactose and N-acetylgalactosamine on the extracytoplasmic surface of the membrane (5).

Marchesi, et. al., (6) sequenced glycophorin A, a major glycoprotein of erythrocyte membrane. It has sixteen oligosaccharide chains on its extracytoplasmic-exposed N-terminal end and a cluster of acidic amino acids on its cytoplasmic C-terminal. Fifteen pentasaccharides are o-linked to serine or threonine while one larger oligosaccharide is N-linked to asparagine. Approximately twenty amino acids, most of which are hydrophobic, are found between the two hydrophilic portions of the polypeptide chain and are believed to span through the bilayer (these could not be arranged in an α-helix because of its short length).

A glycoprotein which accounts for 50% of the protein and all of the nonlipid carbohydrate of the cell envelope of

Halobacterium salinarium has thirty-six di- and trisaccharides linked to threonine near one end of the protein and an asparagine-linked hexacosane-saccharide in an undetermined position in the protein (7). The peptide sequence and the orientation of the protein in the membrane have not been determined. The data indicates an asymmetric distribution of the oligosaccharide on the peptide chain.

The distribution of glycoproteins and proteins in the membrane of Streptomyces albus were studied using non-invasive labelling techniques. Although the majority of the glycoproteins have oligosaccharides that face the extracytoplasmic space, a class of glycoproteins exist which have their oligosaccharide facing the cytoplasm. The intensity of the periodic acid-Schiff stain to the protein stain is greater for this latter class of glycoproteins, indicating that this group has a high sugar to protein content (8).

Treatment of hepatic microsomes with buffered 1M urea results in the selective extraction of a group of proteins together with a portion of the membrane lipids. The major membrane glycoprotein (apparent molecular weight, 171,000) is solubilized by this procedure. It is probably a peripheral protein, and most of it, including its carbohydrate moiety, faces the cytosol. The urea-insoluble fraction comprises 80% of the total protein. It includes a glycoprotein (apparent molecular weight, 35,000) which gives the second most intense stain for carbohydrates. This protein appears to be an integral component of the membrane (9). This experiment also indicates the possibility that a carbohydrate-

enrich glycoprotein faces the cytosol.

Different lipids comprise the two halves of the bilayer of the membrane. For many lipids, e.g.: phospholipids (10), the asymmetry is not complete, glycolipids, however, appear to be asymmetrically distributed. The glycolipids of intact erythrocytes are labeled by reduction with sodium borotritide after galactose oxidase treatment, but the glycolipids of inside-out vesicles are not (11). The carbohydrate chains of both glycolipids and glycoproteins appear to be predominantly or entirely of the extraplasmic half of the membrane. These two classes of compounds can therefore play an important role in surface-mediated processes.

Gangliosides (cerebrosides containing sialic acid) appear to be important in the binding and subsequent activation of membrane-bound adenylate cyclase by glycoprotein hormones and cholera toxin in mammals (12, 13). Cyclic AMP is known to effect the growth properties of many cultured cells (14). In addition, many transformed cell lines have low levels of adenylate cyclase and some have an altered ganglioside composition (15), possibly making the transformed cells less sensitive to physiological control via transmembrane communication mediated by the gangliosides.

There are more known roles for glycoproteins. The transport of anions, glucose and possibly water across erythrocyte membrane appears to be mediated by one or several integral proteins. These proteins appear in one band on a SDS-polyacrylamide gel which also stains positively for carbohydrates (16). Since transport

is a vectorial process, it is not surprising that glycoproteins may be involved.

Glycoproteins are believed to play a pivotal role in cell adhesion and recognition. Roseman (17) hypothesized that the interaction of surface glycosyltransferases and their substrates, the carbohydrate moieties on glycoproteins, is the basis for specific cell adhesion. An enzyme on a surface of a cell adds a sugar to the existing oligosaccharide of a glycoprotein or glycolipid on a neighboring cell. The newly formed oligosaccharide either has a higher or lower affinity for the surface glycosylytransferases of the sugar-donating cell (the relationship can be reciprocal). A higher affinity will produce adhesion between the cells while a lower affinity will permit the cells to detach. Eventually, addition of sugars will increase oligosaccharide-enzyme affinity and produce permanent adhesion between appropriate cells (recognition). This hypothesis is amenable to experimentation.

Many unicellular organisms release acidic glycoproteins or acidic glycoprotein-containing complexes which serve as species-specific agglutinins. The aggregation factor of the sponge, Geodia cydonium, is associated with a particle of molecular weight 2-5 x 10^9 daltons, 74% protein. The aggregation factor has a molecular weight of 20,000 daltons and binds to a surface-bound macromolecular, termed the aggregation receptor. The receptor consists of 81% neutral carbohydrate and 7.5% protein. Sponge cell aggregation will only occur if the receptors are sialylated

and a sialyltransferase has been purified from the high molecular weight complex (18). The overall mechanism appears to be the sialylation of a surface glycoprotein creating an acidic glycoprotein. The acidic glycoprotein, in turn, binds to aggregation factor which is associated with the high molecular weight complex. Since the complex is multivalent, several sponge cells are brought into close proximity. The agglutination factor from the haploid yeast cell, Hansenula wingei type 5 (19) is an acidic glycoprotein and the aggregation factor of the marine sponge, Microciona parthena, is an acidic proteoglycan complex (20).

If sexually active forms of flagellated algae, (+) and (-) gametes, are mixed, the flagella of the two gametes stick together forming large clumps of cells. Eventually pairs of algae break away from the clumps; contact between the pair is only at their flagellar tips. Within minutes the pair fuse and the flagellas which seconds before had been highly adhesive, lose their adhesiveness (21).

The agglutinins of the chlorophycean, Chlamydomonas, are located in a nonpartianlate form, isoagglutinins, and two particulate forms, the gametic flagella (tips) and gametic gamone, a high molecular weight particle cells slough off into the media. The interaction of the flagella of (+) and (-) gametes and gametic gamones (22) or of (+) and (-) isoagglutinins(23) cause an instantaneous agglutination. Since the mating reaction occurs only between gametic flagella of opposite sex, contact must be

based upon a species-specific complementarity that has sex specific components. No contact occurs between vegetative cells (non-sexual cells incapable of forming zygotes) indicating that the mating-type substances are synthesized or unmasked at gametogenesis.

The isoelectric points of the isoagglutinins of Chlamydomonas eugametos and C. moewusii are acidic. The isoagglutinin of C. moewusii contains 5.5% SO_4 and both isoagglutinins contain rhamnose, xylose, arabinose, galactose, mannose and three more unknown sugar components (23).

Gametic gamone, a high molecular weight particle that the cells slough off into the media, can be fractionated into membrane vesicles and mastigonemes (flagella hairs). The aggregating activity is higher in the membrane vesicle preparation than in the mastigoneme preparation (21).

Both vegetative and gametic gamone contain a band on an SDS-polyacrylamide gel of an apparent molecular weight considerably greater than 170,000 daltons which stains for carbohydrate as well as protein. This band comigrates with the major constituent of the flagella membrane and the fractionated membrane vesicles. Since gametic flagella, gametic gamone and its fractionated membrane vesicles, but not the fractionated mastigonemes, show agglutinability, it is likely that glycoproteins associated with the gametic flagellar membrane play a role in the sexual adhesion.

Bosmann and McLean (24, 25) studied the extracellular glycosyltransferases of whole cells or gamones of gametes and vegetative cells of Chlamydomonas moewussi. The specific activities of the various glycosyltransferases of unmixed and

mixed fractions were compared. There is no increase in specific activity when (+) and (-) vegetative components are mixed but there is a several fold increase in all of the specific activities after mixing (+) and (-) gametic components. The largest relative increase is consistently seen for CMP-N-acetyl neuraminic acid, which is similar to the Geodia cydonium aggregation system. It appears that in both systems glycosyltransferase is intimately connected with adhesion.

Freeze fracture indicates that the inner face of vegetative flagella membranes is devoid of particles whereas the gametic flagella inner membranes have an intermediate density of particles along the entire length of the flagella (26). These particles can possibly be involved in the adhesion reaction, although their localization in the inner half of the membrane and their distribution along the entire length of the flagella rather than in the flagella tip might indicate otherwise.

The gametes of Chlamydomonas have more protein aggregates of unknown function in their flagellar membrane, and have an increase in flagellar glycosyltransferase activity when (+) and (-) gametes are mixed. Cell adhesion in Chlamydomonas is not understood on a molecular basis. The sugar composition of the soluble isoagglutinins of Chlamydomonas has been qualitatively studied. Also, the addition of neuraminic acid to an endogenous glycoprotein acceptor is possibly involved in flagellar adhesion. No work has been done on the chemical characterization of the 170,000 dalton glycoprotein found in large quantities in vegetative and gametic membranes and gamones, although recently U. Goodenough

found that there are several bands on SDS-gel electrophoresis when analyzed on appropriately cross-linked gels (personal communication).

Chen and Haines (27) isolated and purified the flagella membrane and mastigonemes of the phytoflagellate, Ochromonas danica. The major membrane protein components have apparent molecular weights of 54,000, 47,000, 35,000, 31,000 and 28,000 on SDS-polyacrylamide gels. There is a large protein band at the origin which does not migrate into the gel. The bands were not stained for carbohydrates because of the difficulty in obtaining enough pure membrane to be visualized by the relatively weak periodic acid-Schiff stain.

There is electron-microscopic evidence that the flagella membrane contains glycoproteins. Thiery's method (periodic acid treatment followed by a silver reaction) for the visualization of glycoproteins stains the flagella membrane, mastigonemes and cell plasma membrane of Ochromonas (28). Periodic acid treatment followed by phosphotungetic acid-chromic acid selectively stains the plasma membrane and flagella membrane of Ochromonas danica (29). Both staining techniques are specific for glycoproteins and were used to selectively stain membranes of other algae.

The flagella membrane of O. danica appears to contain a small amount of glycolipids as detected by diphenylamine on TLC plates. The major lipids are sterols, free fatty acids, chlorosulfolipids, and a highly polar unknown which constitutes 83% (w/w) of the total lipid extract and is 35% carbohydrate (30). The chlorosulfolipids are derivatives of 1, 14-docosanediol-1, 14-disulfate and of 1, 15-tetracosanediol-1, 15-disulfate with

zero to six chlorine atoms replacing hydrogen atoms on the saturated hydrocarbon (31).

The flagella membrane of Ochromonas danica appears to consist primarily of an acidic lipid, the chlorosulfolipids, and a small amount of uncharacterized sugar-containing lipids. Knowledge of the composition and organization of the glycolipids and a large amount of unknown which is neither protein nor lipid is important.

The mastigonemes (flagellar hairs) of O. danica are of two types, fibrous and tubular. The fibrous mastigonemes are of small diameter (50-100 Å) and 1-3 microns long and are found on both the long and short flagella of O. danica. The tubular mastigonemes which are about 200 Å in diameter, and always one micron long, possess a tapered basal region attached to a micro-tubular-like shaft. Each of the tubular mastigonemes has two sets of lateral filaments, a shorter one (350 Å) positioned around the full diameter in 5-6 rows and long lateral filaments (2000 Å), intermittently placed (32).

The tubular mastigonemes of O. danica appear to pass through the membrane and are attached to the central tubules (33). The mastigonemes are found in two unbalanced files on opposite sides of the flagellum; one file consists of single mastigonemes while the other consists of clusters of two or more mastigonemes separated from adjacent clusters by a distance of 3000 Å or less (32). Forward movement of the cell occurs because the displacement of medium towards the organism by passive movement of the mastigonemes is relatively greater than the displacement of

medium away from the organism by the action of the flagellum alone (34).

The biogenesis of the mastigonemes can be examined by an electron-micrograph sequential study after flagella amputation. Presumptive mastigonemes first appear in the perinuclear space, an area bounded by the nuclear envelope and a sheath of endoplasmic reticulum which surrounds the nucleus and chloroplasts. The objects seen in this space lack lateral filaments and have a somewhat smaller diameter than mature mastigonemes (32, 35). The mastigonemes are next seen in the Golgi complex and here one sees lateral filaments attached. Furthermore, the mastigonemes are found enclosed in sacs. There appears to be a segregation of sacs that contain single mastigonemes from those that contain clusters of three mastigonemes. The sacs that contain single mastigonemes are on opposite ends of the Golgi complex from the sacs that contain three mastigonemes. The sacs containing mastigonemes then appear between the Golgi complex and the cell membrane adjacent to the cell membrane. The electron microscopic evidence suggests that mature mastigonemes are deposited on the membrane near the flagella base (35).

The above route, determined by electron microscopy, for the biosynthesis of extraflagellar mastigonemes is similar to the more extensively studied pathway for the synthesis, sequestering and discharge of secretory proteins. These proteins are attached to the membranes of the endoplasmic reticulum and vectorially discharged into the lumen of the rough endoplasmic

reticulum and then to the smooth endoplasmic reticulum, to the Golgi complex, and to Golgi derived secretory vesicles which fuse with the plasma membrane and discharge the secretory proteins. The carbohydrate moieties of the secretory glycoproteins are added in stepwise fashion within the organelles with the terminal glycosylation occurring in the Golgi complex (36).

The mastigoneme preparation of O. danica on SDS-gel electrophoresis contains three major bands, all of which are glycoproteins. In addition, a carbohydrate band is seen near the bromphenol tracking dye (27). The characterization of the carbohydrate of the glycoproteins and of the oligosaccharide will help in the determination of site of synthesis of each component. Presumably, some of the carbohydrate of the glycoproteins as well as the entire peptide chain are synthesized in the perinuclear space and the terminal sugars are added in the Golgi complex. Immature mastigonemes between the Golgi apparatus and the cell membrane and mature mastigonemes do not appear identical in electron micrographs (32, 35). Thus the mastigonemes must be further modified after leaving the Golgi complex.

Among the sugars found in O. danica's flagella are rhamnose, fucose (both are L-6-deoxyhexose) and xylose. Rhamnose is found in many algae, plants, and some bacteria, yeast, and invertebrate, although it is possible that it is more abundant in non-plant sources but its presence was overlooked. Rhamnose and xylose are often found in the same molecule, but there has been only

one report (a diatom) in which a polysaccharide contains both rhamnose and fucose. The isoagglutinins of Chlamydomonas and the surface receptor of the sponge, G. cydonium (38) are the only known glycoproteins to contain rhamnose (Table I).

Rhamnose is also found in several acidic algae polysaccharides,some of which are sulphate-containing. A rhamnose-2-sulphate containing polysaccharide was characterized in Ulva lactuca. It is interesting that Chlamydomonas isoagglutinin, the flagella membrane of O. danica and a polysaccharide of Ulva lactuca contain both rhamnose and sulphate. The sulfate content of the isoagglutinin of Chlamydomonas and the O. danica flagella membrane are strikingly similar (37). In Klebsiella K-type 72, a bacteria, rhamnose is found linked by ketal linkages to pyruvic acid; pyruvic acid is also found in the agar of many algae.

Xylose is also found in acidic algal polysaccharides, many of which are sulphated. There is no evidence for the presence of a sulphated xylose. Xylose is usually 1, 2-linked, 1,4-linked or the terminal sugar in neutral and acidic compounds. A protein-containing heteropolysaccharide of Sargassum linifolium is both sulphated and contains fucose and xylose. Dermatan sulphate, chondroitin-4-sulphate, chondroitin-6-sulphate and heparin are glycoproteins that contain xylose at the reducing end of the oligosaccharide. Fucose and xylose are constituents of the enzyme, horseradish peroxidase, and xylose is found in glycoproteins of yeast and of the flour of apple seeds (Table II).

Both rhamnose and xylose are found in algae and plants predominantly in cell wall components (celluloses and hemicelluloses) or extracellular polysaccharides (agar). Rhamnose has not been found in vertebrates and is found in only two glycoproteins. Xylose is found in both invertebrates and vertebrates and in many glycoproteins. Both rhamnose and xylose are found in many polysaccharides. Recent evidence indicates that all polysaccharides have a protein core to which the carbohydrate chains are attached, e. g.: glycogen (39).

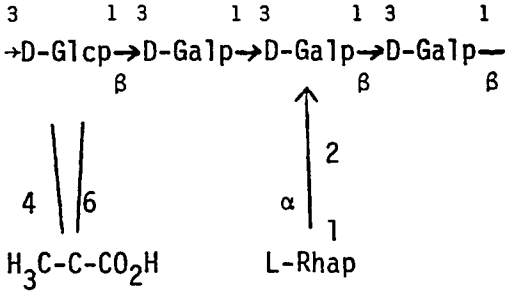
In this work the neutral and amino sugar composition of the membrane, lipids and mastigonemes of the Ochromonas danica flagella are investigated together with those of the entire flagella. Approximately ten molar percent of the lipids have not been characterized and appear to give a positive test with diphenylamine (30). These lipids appear to be sugar-containing diglycerides. The other sugars appear to occur as glycoconjugates on the membrane and mastigonemes (28, 29). The mastigonemes consist primarily of glycoproteins stacked in a helical array (32). These structures serve as passive "oars" and provide a large surface area which provide a beating flagella with greater contact with the aqueous medium. The glycoproteins of the membrane are presumably involved in chemotactic activities and cell adhesion.

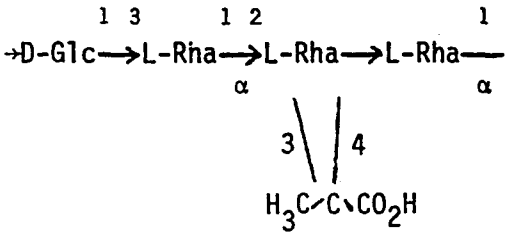
Table I

Rhamnose-Containing Compounds

Species	Component	Composition	Reference
Algae:			
Chlorophyceae (green algae)			
Chlamydomonas moewusii	Acidic isoagglutinin	5.5% SO_4, ara, xyl, gal, man, rha and three other components	23
Chlamydomonas eugametos	Acidic isoagglutinin	Ara, xyl, gal, man, rha and three other components	23
Chlamydomonas reinhardii	Medium macromolecules	Six proteins (75%) and sugar (25%) originating from cell walls. Ara (20%), xyl (3%), gal 27%), glc (9%), man (30%) and rha (6%)	40
Codium fragile (green seaweed)	Sulfated polysaccharide	SO_4, ara, xyl, gal, glc, man and rha	41
Ulva lactuca (green seaweed)	Sulfated polysaccharide	GlcA (1→4)-L-rha-2-sulfate rhap (1→4)-xylp (1→3)-glcp glcAp (1→4)-xylp glcAP (1→4)-rhap (1→3)-glcAP (1→3)-xylp glcp (1→3)-xylp	42

Species	Component	Composition	Reference
Chlorella sp.	Polysaccharide	90%, 1, 3-glc and smaller amounts of ara, gal, man, rha and N	43
Ulva lactuca	Hemicellulose	Major constituents are ara, gal, rha. xyl is a minor constituent.	44
Enteromorpha sp.	Hemicellulose	Major constituents are xyl, rha. Glc is a minor constituent.	44
Chorella pyrenoidosa	Hemicellulose	Major constituents are gal, man, and ara. Xyl and rha are minor constituents.	44
Chrysophyceae (yellow algae)			
Ochromonas malhamensis	Non-cellulosic, non-chitinous matrix material of lorica	Xyl, gal, glc, man, (glucosamine) and rha	45
Euglenephyceae			
Euglena gracilis	Pellicle	80% protein, 12% lipid, 17% neutral sugars, 0.4% hexosamine. Xyl, gal, glc, man, fuc and rha.	46
Rhodophyceae (red algae)			

Species	Component	Composition	Reference
Bactrachospermum sp.	Gelatinous polysaccharide	Xyl, D- and L-gal, man, glcA, rha, 3-O-methyl-gal, 3-O-methyl-rha	47
Bacillariophyceae (diatom)			
Chaetoceros affinis var Willei	Branched extracellular polysaccharide	Rha is found only in outer portion while gal and fuc are found in both outer and inner	48
Bacteria			
Clostridium	Surface layer glycoprotein	One rha and 2 or 7 hexoses per 140,000 daltons	49
Streptococcus	Glycerophosphate-containing cell wall polysaccharide	Glc, glcNAc and rha	50
Streptococcal group P		gal, glc, galactosamine, glucosamine and rha	51
Klebsiella K-type 56	Capsular polysaccharide		52

Species	Component	Composition	Reference
Klebsiella K-type 72	Capsular polysaccharide		53
Serratia marcesens	Capsular polysaccharide	Glc, man, uronic acid and rha	54
Myxococcus	Lipopolysaccharide	Gal, glc, man, NAcgal, NAcglc and rha	55
Yeast			
Alternacia solani	Extracellular polysaccharide	Gal, glc, man, glcA and rha	56
C. trichordes	Water soluble polysaccharide from cell walls	Gal, glc, man and rha	57
Invertebrates			
Ommastrephes sloani (squid)	Liver	Ara, xyl, gal, glcA, fuc and rha	58

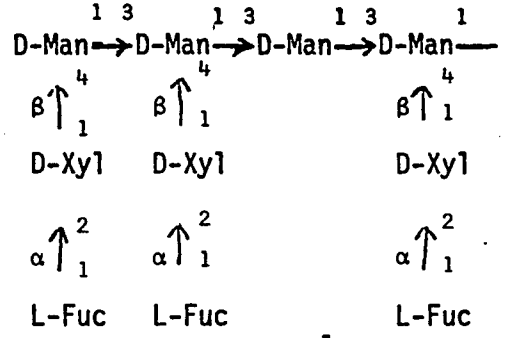
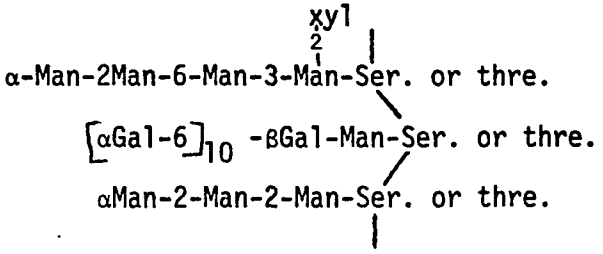
Species	Component	Composition	Reference
Ocypode platytaris (shore crab)	Hemolymph	Rha	59
Geodia cydonium (sponge)	Surface receptor for aggregation factor	7.5% protein and 81% neutral carbohydrate. Arabinose, gal, rha.	38

Table II

Xylose-Containing Compounds

Species	Component	Composition	Reference
Algae			
Chlorophyceae (green algae)			
Chlamydomonas	Cell wall	Ara (24.8%), xyl (2.2%), gal (19.6%), glc (1.8%), man (1.4%), uronic acid (5.3%), unknown sugars (2%), hydroxyproline (3.0%), other amino acids (7.2%), lipid (6.9%), ash (10.2%), water (8.0%). The amino acid composition of this cell wall is similar to Volvox sheath and tomato cell walls.	60
Phaeophyceae (brown algae)			
Sargassum linifolium	Sulfated polysaccharide	Xyl, gal, man, glcA and a protein moiety	61
Sargassum pallidum	Polysaccharide	Xyl,-fuc, fuc-xyl, fuc-gal, xyl-gal, xyl-fuc-gal, $(gal)_n$-gal	62
Asophyllum nodosum	Glucuronoxylofucan	GlcA-fuc and fuc-xyl. Xyl is either end group or 1, 4-linked. Fucose is found at end group, 1, 2-linked, 1,2-linked-4-sulfate, 1,3-linked-4-sulfate, and 1,2,3,4-linked or sulfate.	63

Species	Component	Composition	Reference
Rhodophyceae (red algae)			
Anatheca dentata	Agar-like sulfated polysaccharide	1,2-linked and 1,4-linked xyl, trisulfated-L-gal and 4,6-O-(1-carboxyethylidene)-D-gal	64
Cyanophyceae (blue green algae)			
Anabaena cylindrica	Polysaccharide from the envelopes of heterocysts and spores	The linear portion and branch points contain glc and man, while the terminal sugars are xyl, gal, glc and man	65
Higher Plants			
Scenedesmus obliquus	Cell wall	Ara, xyl, gal, glc, man and fuc	66
Phaseolus aureus	Hemicellulose of cell wall	Xyl, gal and glc. Glucose forms the backbone	67
Armoracia rusticana (horse-radish)	Isoenzymes of peroxidase	Ara, xyl, man, and fuc	68
Apple	A glycoprotein from flour of apple seeds, with a molecular weight of 50,000 daltons	Xyl, gal, glc, man and glucosamine	69

Species	Component	Composition	Reference
Bacteria			
Bacillus subtilis	Slimy substances	Xyl, glc and man	70
Pseudomonas oleovorans	Extracellular polysaccharide	Xyl, gal and glc	71
Yeast			
Fomes annosus	Polysaccharide in fruit bodies		72
Cryptococcus laurentii	Glycoprotein from the envelope		73

Species	Component	Composition	Reference
Others			
Vertebrates	Dermatan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, and heparin	β-D-Glc-(1→3)-β-D-Gal-(1→3)-β-D-Gal-(1→4)-D-xyl-O-Ser(Protein)	74
Dictyostellium discoidium	The surface sheath of tipless aggregates, of migrating slugs and trails of sheath.	Xyl, gal, glc, man, NAcglc and fuc	75

EXPERIMENTAL PROCEDURE

Cultures - Ochromonas danica was grown in the chemically defined medium of Aaronson and Baker (76) at 23° in darkness. Inoculations were conducted in ambient light.

Flagella Detachment and Isolation - Cultures of O. danica were harvested five days after inoculation by centrifugation for 15 min. at 4° and 300 x g in a Sorvall 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 kept cool for 1 hour before deflagellation. Flagella were detached by agitation in a 2.5 cm x 10 cm centrifuge tube for 8 x 3 sec. at top speed in a Vari-Whirl mixer (Van Waters & Rogers Scientific). Intact and deflagellated cell bodies were then removed from the medium by centrifugation for 10 min at 208 x g at 4° several times in a SS-34 rotor of Sorval RC2-B centrifuge. The supernatant was centrifuged at 13,300 x g for 20 min. The pellet obtained was milky white (27).

The pellet was either used in its present condition for the isolation of flagella membrane and mastigonemes or it was washed prior to sugar analysis by gas-liquid chromatography or spectrophotometrically. The pellet was resuspended in either thirty volumes of 100 mM sodium acetate, pH 3.9, (for gas-liquid chromatography) or thirty volumes of medium where the glucose had been replaced with mannitol (for anthrone assay). The flagella was centrifuged at 13,300 x g for 20 min at 4° in a SS-34 rotor. The pellet was resuspended in thirty volumes of the appropriate

wash solution and recentrifuged three times. The flagella were collected as a compact pellet after centrifugation at 39,000 x g for 20 min at 4°.

Isolation of Flagellar Lipids and Lipid-Extracted Flagella - Thirty volumes of chloroform: methanol (2:1) were added to the flagella pellet which had been washed with 100 mM sodium acetate. The cloudy solution was centrifuged for 15 min at 39,100 x g. The clear supernatant was decanted. The pellet was resuspended in thirty volumes of chloroform: methanol (2:1) and recentrifuged. The supernatants were pooled. The pellet constitutes the preparation of lipid-extracted flagella.

The lipid extracts were diluted with absolute ethanol to a final concentration of 20% (v/v). The solution was evaporated to dryness under reduced pressure at 40°. The dry sample was extracted five times with 2 ml chloroform which had previously been bubbled with nitrogen. The pooled chloroform washes constitute the lipids of the whole flagella.

Flagella Membrane and Mastigoneme Isolation - To remove the membrane from the axonemes, the unwashed whole flagella pellet was resuspended in thirty-five volumes of 10 mM Tris-HCl, pH 7.5, 4 mM MgCl2, ImM mercaptoethanol (TMM) and left over night in the cold room stirring magnetically. The solution was vortexed at maximum speed for 30 min, diluted with one volume of TMM and centrifuged at 17,300 x g for 15 min. The supernatant was decanted and recentrifuged at 17,300 x g for 15 min at 4°. The pellet was discarded and the supernatant was again centrifuged at

22,000 x g for 1 hr. The resulting pellet of mastigonemes was resuspended in a small volume of TMM. The supernatant was centrifuged at 22,000 x g for 1 hr. The supernatant was centrifuged at 113,700 x g for 75 min in a Beckman Ultracentrifuge Model L2-65B (60 Ti rotor). The pellet obtained was resuspended in a small volume of TMM and respun at 24,150 x g for 15 min in a Beckman Ultracentrifuge Model L2-65B (50 rotor). The pellet consists of mastigonemes and membranes. The supernatant was spun at 96,600 x g for 75 min. The pellet is approximately 95% pure membrane as determined by electron microscopy (37).

Methanolysis of Flagella Fractions and the Separation of Methyl Glycosides from Diols and Methyl Esters of Fatty Acids - Dry 1.5 N methanolic HCl (Supelco) was added to each dried fraction, the samples are sealed tightly with teflon-lined screw caps and heated at 67° for 16-24 hrs (77). A molar excess of silver carbonate (Alfa) 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, concentrated to a small volume and extracted three times with 1 ml hexane. The lower phase was concentrated to dryness and 1 ml of water and 1 ml of chloroform was added. The water phase was washed three times with 1 ml chloroform. The water phase contains the methyl glycosides and the chloroform wash contains most of the diols (Scheme I).

Preparation of Pertrimethylsilyl Derivatives of Diols and Methly Glycosides - Trimethylsilylimidazole in silylation grade

Scheme I

Separation of Methyl Esters of Fatty Acids, Chlorodiols and Methyl Glycosides formed from Methanolic · HCl Hydrolysis of Flagellar Fractions

Whole flagella or membrane (1-5 mgms)

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

Upper Phases

Predominantly methyl esters of fatty acids plus perchlorinated diols.

Lower Phase

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

Upper Phase

Methyl glycosides

Lower Phase

Chlorodiols

pyridine (Pierce Chemical Company) was added to either dried diols or methyl glycoside samples and the solution heated in a sealed tube at 67° for 15 min before injection into the gas chromatograph.

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

Trimethylsilyl derivatives of methyl glycosides were analyzed with several different column conditions: (1) 90° for 10 min followed by a linear program of 2°/min to 250°, or (2) a linear program of 0.5°/min from 140° to 200° (78) or (3) 160° isothermal (77).

Trimethylsilyl derivatives of diols were analyzed at either (1) 90° for 10 min, followed by a linear program of 2°/min till 280° or (2) 212° for 2 min followed by a linear program of 6°/min till 267° (79).

Thin Layer Chromatography of Methyl Glycosides and Diols - The methyl glycosides of flagella fractions and prepared methyl glycoside standards were spotted on an analytical silica gel plate (Brinkman) and run in benzene: ethanol: water: concentrated ammonia (28%) (100:100:15:1), a modification of a solvent system of Hay, et. al. (80). The plate was visualized by spraying with 3% (2/v) H_2SO_4 - 25% (w/v) $NaHSO_4$, followed by heating at 450°.

Fractions that contained diols were spotted on analytical silica gel G plates (Analtech) and run in chloroform: methanol: water (100:55:4) (79). The spots were visualized by spraying with 3% (w/v) H_2SO_4 - 25% (w/v) $NaHSO_4$ and charring at 450°.

Thin Layer Chromatography of Flagella Lipids - Analytical silica gel G plates (Analtech), 20 cm x 20 cm, were activated at 60° for 1 hour, cooled in a dessicator and developed in the first direction with chloroform: methanol: concentrated ammonia (28%) (65:35:5). Chromatograms were dried for about 10 min at 60°, cooled in a dessicator and then developed in the second direction with chloroform: acetone: methanol: glacial acetic acid: water (80:32:16:16:8) (81). The plate was either sprayed with 3% (2/v) H_2SO_4 - 25% (w/v) $NaHSO_4$ and charred to visualize all the lipids or it was sprayed with the molybdenum trioxide reagent which reacts with phospholipids.

Molybdenum Trioxide - Equal volumes of solution A (40.11 g molybdenum trioxide in 1 l. 25N 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.) were mixed and carefully diluted with 2 volumes of water. The solution was sprayed as a fine mist on the plate and phospholipids appeared as blue spots on a white background (82).

Hexose Determination by the Anthrone Assay - Dry samples were resuspended with 2 ml water in 19 x 150 mm test tubes and 0.5 ml of 2% (w/v) anthrone in ethyl acetate was added. Five milliliters of concentrated sulfuric acid was carefully layered

on the bottom of the tubes and the solutions were vortexed until the anthrone dissolved. The solutions were read at 620 nm after 10 min (83).

Amino Sugar Colorimetric Assay - Flagella fractions were dried and 0.25 ml of water and 0.25 ml of 8N hydrochloric acid were added to each tube. The tubes were closed tightly with a teflon-lined cap and heated in a boiling water bath for 5 hrs. The tubes were placed at 4° for 1 hr, placed in a vacuum desiccator and connected to a water aspirator overnight. The samples were placed in a drying pistol, which was kept at the boiling point of 95% ethanol (75°) and attached to a vacuum pump. After at least 6 hrs the samples were resuspended in a small volume of water and dried in the drying pistol. This was repeated till the samples had a pH of approximately three.

The dry samples were resuspended in 0.6 ml water and 0.1 ml of 1.5% (v/v) acetic anhydride in acetone and 0.5 ml of 0.7 M boric acid, pH 9.1, 4% (w/v) with respect to glycerol were added. The tubes were closed and heated in a boiling water bath for 3 min. After cooling 6.0 ml of dimethylaminobenzaldehyde solution (10.0 gm DMAB (Fisher) in 12 ml concentrated hydrochloric acid, plus 88 ml glacial acetic acid, diluted with 9 volumes of glacial acetic acid before use) was added and the tubes heated at 37° for 20 min. The rose violet color was read at 585 nm immediately after cooling (84).

The Azure A Colorimetric Assay for Sulfolipid - The procedure of Kean (85) 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 0.5N H_2SO_4 and 1.0 ml of Azure A solution (40 mg in 5.0 ml of 0.5N H_2SO_4 diluted to 100 ml with water). The tubes were capped, vortexed for 30 sec and centrifuged (300 x g for 5 min). the absorbance at 645 nm of the lower phase is a molar measurement using SDS as a standard. It should be noted that the measurement of O. danica sulfolipids (with two sulfates on the molecule) must be halved to obtain molar quantities.

Hexose Determination with Sulfuric Acid-Cysteine - Flagella fractions and cell bodies were dried. Water (0.25 ml) and 8N hydrochloric acid (0.25 ml) were added. The tubes were closed tightly with a teflon-lined cap and heated in a boiling water bath for 5 hrs. Samples were neutralized by the addition of 4N sodium hydroxide or by passage through a mixed-bed ion-exchange resin (Dowex 1 and Dowex 50) column (1 cm x 10 cm).

The samples were concentrated to a volume of 200 µl and were cooled in an ice bath. Sulfuric acid (30 N, 0.9 ml) was added. The solutions were shaken carefully and allowed to come to room temperature. They were then placed in a boiling water bath for exactly three minutes, and cooled to room temperature under running tap water. Twenty microliters of 3% (w/v) cysteine (freshly prepared) were added and the shaken solutions placed in the dark for 30 min. at room temperature. The difference in absorbance between 415 and 380 nm of the solutions were used to determine the hexose content (86).

6-Deoxyhexose Determination With Sulfuric Acid-Cysteine - Flagella fractions and cell bodies were dried. Water (0.25 ml) and 8 N hydrochloric acid (0.25 ml) were added. The tubes were closed tightly with a teflon-lined cap and heated in a boiling water bath for 5 hrs. The samples were neutralized by addition of 4 N sodium hydroxide or by passage through a mixed-bed ion-exchange resin (Dowex 1 and Dowex 50) column (1 cm x 10 cm).

The samples were concentrated to a volume of 200 µl and were cooled in an ice bath. Sulfuric acid (30 N, 0.9 ml) was added and the solutions were shaken carefully. The solutions were allowed to reach room temperature. They were then placed in a boiling water bath for 5 mins and cooled to room temperature under running tap water. Twenty microliters of 3% (w/v) cysteine (freshly prepared) were added and the solutions were placed in the dark for 3 hrs at room temperature. The difference in absorbance between 396 and 427 nm of the solutions were used to determine the 6-deoxyhexose content (87).

Amino Sugar Determination with an Amino Acid Analyzer - A preparation of flagellar membrane (330 µg) was dried in a desiccator. Water (0.25 ml) and 8 N hydrochloric acid (0.25 ml) were added. The tube was closed tightly with a teflon-lined cap and heated in a boiling water bath for 5 hrs. The sample was concentrated to dryness on a rotary evaporator and resuspended in a small volume of 0.35 M sodium citrate, 0.3 M borate, 15% (v/v) n-propanol. A nanogram of ε-aminocaproic acid was added as an internal standard.

An aminex A-4 resin (Biorad) in 0.35 M citrate-borate, 15% (v/v) n-propanol was used to separate the amino sugars and the aminocaproic acid. The column (55°C) was eluted for 15 mins at a flow rate of 60 ml/hr with 0.35 M sodium citrate, 0.3 M borate, and 15% (v/v) n-propanol. This was followed by 0.35 M sodium citrate, 0.3 M borate, 0.2 M sodium chloride, and 15% (v/v) n-propanol. The amino sugars and internal standard were detected spectrophotometrically after reaction with ninhydrin (88).

Protein Assay - The concentration of protein was determined by the method of Lowry, et. al. (89), using lipid-extracted bovine serum albumin (Sigma) as a standard.

Weighing of Flagella Fractions - Microsamples (up to 10 milligrams) were weighed on an electrobalance (Ventron Instruments Corp.).

RESULTS

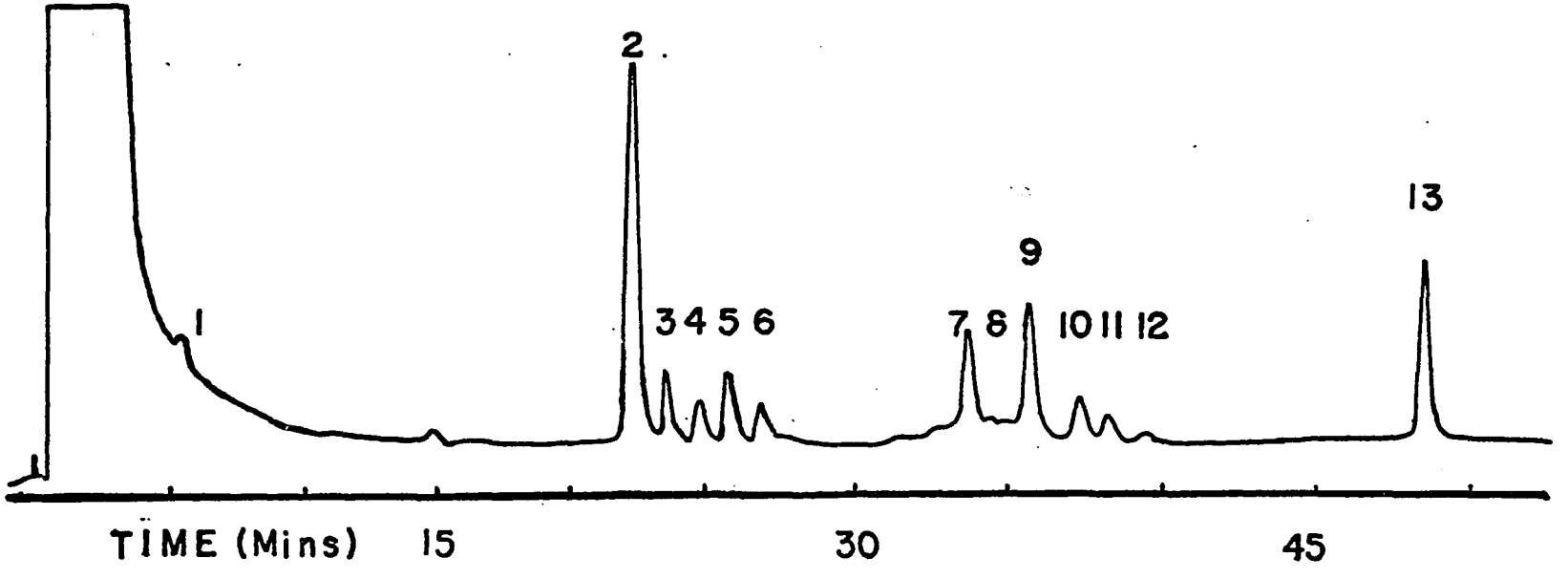
Gas Liquid Chromatographic Determination of the Sugar Composition of O. danica Flagella Fractions - The flagella (membrane) has chlorosulfolipid, fatty acid-containing lipids and glycoconjugates (30), all of which are hydrolyzed by 1.5 N methanolic HCl. The hydrolyzed products of these compounds were separated as shown in Scheme I. It was necessary to separate the diols from the methyl glycosides because the trimethylsilyl derivatives (TMS) of (non, mono and di-chlorinated) diols and methyl glycosides had similar retention times on gas liquid chromatography. Thin layer chromatography showed that the chloroform wash contained only diols. The hexane wash contained methyl esters of fatty acids and polychlorinated diols.

The whole flagella were separated from the cell bodies and isolated by centrifugation in the presence of growth medium, which contains glucose. The whole flagella were, therefore, resuspended and recentrifuged four times in thirty volumes of 100 mM sodium acetate. This procedure diminished only the glucose content; it did not affect the amounts of the other sugars. The flagellar membrane and mastigonemes are not washed with sodium acetate because the glucose is effectively removed in their isolation by multiple washings with TMM.

Figure 1 shows a typical glc of the methyl glycosides of a flagellar fraction. The molar percent neutral sugar composition

Figure 1

Separation of a standard mixture of monosaccharides of mastigonemes subjected to gas chromatography as trimethylsilyl derivatives of methyl glycosides. Peaks are numbered in their order of emergence and are identified as follows: glycerol 1; rhamnose 2; fucose 3, 4; xylose 5, 6; mannose 7; galactose 8, 9, 10; glucose 11, 12 and myo-inositol (internal standard) 13. Column program was 110° for 10 mins followed by 2° per min.


2
13
9
1
3 4 5 6
7 8
10 11 12
TIME (Mins)
15
30
45

of the flagellar fractions are shown in Table III. Not included in the Table is arabinose which appeared in trace amounts. In addition, there were several peaks which had retention times similar to the trimethylsilyl derivatives of methyl mannoside and methyl galactoside under three different column conditions. The unknown peaks together never constituted more than 5-10% of the total area and have not been identified.

The sugar composition of whole flagella and mastigonemes are virtually identical. It appears that the bulk of the carbohydrate of the whole flagella is that of the mastigonemes. The dominant sugar in the mastigonemes as well as in the membrane glycoproteins is rhamnose.

Chen et. al. (30), found no evidence for phospholipid in the flagella membrane. Inositol seen in the lipid extract (Table III)may well originate from phosphatidyl inositol. A check of the lipid extract by thin layer chromatography revealed a small amount of phospholipid. The phospholipids probably are not present in the flagella but originate from some contamination in the organelle preparation.

Identification of Sugars in the Gas Chromatograph - The gas liquid chromatography (glc) of sugars is not as simple as that of most compounds. The sugars are generally chromatographed after two derivatization steps; first methyl glycosides are formed and these are then converted to their trimethylsilyl ethers of those methyl glycosides. Each sugar generally chromatographs as several peaks due to the formation of pyranosides and furanosides as

TABLE III

MOLAR PERCENT NEUTRAL SUGARS IN DIFFERENT FLAGELLAR FRACTIONS

SUGAR	WHOLE FLAGELLA	MASTIGONEMES	MEMBRANE	LIPIDS
Glycerol	1	1	>1	95
Rhamnose	46	48	35	N.D.
Fucose	11	13	22	N.D.
Xylose	13	12	9	N.D.
Mannose	10	8	6	N.D.
Galactose	17	13	22	2
Glucose	3	3	6	N.D.
Inositol	N.D.	N.D.	N.D.	3

N.D. Not Detected

well as α- and β-methyl glycosides. The relative ratios of each of these peaks are constant for each sugar and are defined by the derivative preparation conditions. The relative amounts in addition to the relative retention times can therefore be used for characterization (78). The retention times were compared to Bhatti, et. al. (78), and Sweeley and Tao (77) (two different chromatographic conditions). Additionally standards were co-chromatographed. The only sugar which was not studied by the above workers was rhamnose. Rhamnose has only one peak in the glc. Its assignment was further verified by thin layer chromatography in several chromatographic systems (80). In some of these systems its relative position changed with respect to other known sugars.

It is possible that the minor peaks in the gas liquid chromatograms are trimethylsilyl derivatives of mannosamine and galactosamine. Bhatti, et. al. (78), report that these substances have retention times similar to the trimethylsilyl derivatives of methyl mannoside and methyl galactoside. Mannosamine and galactosamine are formed from their N-acetyl amino sugars during methanolic HCl treatment (90). Broad peaks, in the appropriate region, were seen on glc with authentic amino sugars. Thin layer chromatography of the methyl glycoside phase of whole flagella shows the presence of small amounts of amino sugars (Figure 2).

The Quantification of the Sugars by Gas Liquid Chromatography - The percent of total neutral sugar in each fraction was estimated by gas liquid chromatography, Table IV. The gas liquid chromatogram

Table IV

Weight Percent of the Total Neutral Sugars in the Different Flagellar Fractions

Fraction	Percent (x100)
Whole Flagella	15
Membrane	1
Mastigonemes	17

was calibrated with injections of known quantities of hexa-trimethylsilyl myo-inositol. The peak was cut out and the weight of the single peak was divided by the number of moles of C - H bonds in the injection (in the compounds studied, only the C - H bonds are detected with flame ionization).

The membrane has a lower content of neutral sugar. The ratio of lipid to protein of the membrane is 3.1:1.0 (30). The lipid extract appears to contain few glycoconjugates and makes a negligible contribution to the sugar composition of the membrane, Table III.

Colorimetric Assays of Hexoses on Flagella Fractions - Hexoses form a blue-colored compound (λ_{max} = 620 nm) after reacting with anthrone and sulfuric acid (83). Hexoses form a yellow-colored compound (λ_{max} = 415 nm) after reacting with sulfuric acid, followed by cysteine (86). 6-Deoxyhexoses (fucose and rhamnose) also form yellow-colored compounds (λ_{max} = 400 nm) under different reaction conditions (87). Flagella fractions and deflagellated cell bodies contained an unknown substance which produced a dark brown colloidal solution in the presence of sulfuric acid. Acid hydrolysis of flagella fractions and deflagellated cell bodies and extraction of the acid hydrolysate with hexane prior to neutralization with sodium hydroxide or passage through a mixed-bed ion-exchange resin column did not remove the unknown. The product of the unknown and sulfuric acid and cysteine was not extractable with hexane or chloroform.

Estimation of the Amino Sugar of the Whole Flagella and Flagella Membrane - The molar extinction coefficients of different

amino sugars are not identical in the colorimetric assay. Galactosamine · HCl was used as the standard in this study. The "equivalent galactosamine · HCl" content of the whole flagella and flagella membrane was 0.4% (w/w) and 2.2% (w/w), respectively. The amino sugar composition of the flagella membrane was also determined on an amino acid analyzer. Glucosamine was found (not galactosamine) and it constituted 0.3% (w/w) of the flagella membrane. The two analyses of the amino sugar content of the flagella membrane are in close agreement because the molar extinction coefficient of glucosamine · HCl in the colorimetric assay is three times that of galactosamine · HCl. The "equivalent glucosamine · HCl" content of the flagella membrane is therefore 0.7% (w/w). The sugar and protein composition of the membrane is shown in Table V.

Thin Layer Chromatography of Methyl Glycosides - Thin layer chromatography (tlc) confirmed that the major neutral sugar in the whole flagella was rhamnose (Figure 2). The tlc also clearly shows the presence of all the identified sugars and the absence of N-acetyl glucosamine and arabinose in the whole flagella.

The completeness of the hydrolysis with 1.5 N methanolic HCl and the purification of methyl glycosides was checked by tlc (Figure 3). Lane 8 was spotted with 200 micrograms of hydrolyzed digitonin (29% (w/w) galactose, 29% (w/w) glucose and 12% (w/w) xylose) and lane 9 was spotted for comparison with the three sugars in the same molar proportion found in digitonin. The plate shows that the hydrolysis was complete.

Table V

Composition of the Flagella Membrane

Component	Percent (w/w)
Neutral sugars	1.0
Amino sugar[a]	2.2
Glucosamine[b]	0.3
Protein	25.0

The amount of neutral sugars were estimated on a gas liquid chromatogram which was calibrated with hexatrimethylsilyl inositol. The $(TMS)_6$ inositol peak was cut out and the ratio of the weight of the recording paper to moles of C - H bonds was calculated. Amino sugar[a] was determined as equivalent galactosamine · HCl by a colorimetric assay (84). Amino sugar[b] was determined on an amino acid analyzer. Protein was estimated by the Lowry method (89), using lipid-extracted bovine serum albumin as a standard.

Figure 2

Thin layer chromatograms of methyl glycosides of whole flagella and authentic sugars. Lane 1, D-galactosamine·HCl; 2, D-mannosamine·HCl; 3, methyl-D-galactoside; 4, methyl-D-glucoside; 5, methyl-D-mannoside; 6, methyl-D-xyloside; 7, methyl-L-fucoside; 8, methyl-L-rhamnoside; 9, combination of lanes 1 througn 8 plus methyl-N-acetyl-D-glucosaminide; 10, combination of lanes 1 through 8; 11, lipid-extracted whole flagella; 12, combination of lanes 1 to 8 plus methyl-D-arabinoside; 13, methyl-N-acetyl-D-glucosaminide; 14, methyl-D-arabinoside. The plate was developed by one ascending run in benzene: ethanol: water: concentrated ammonia (100:100:15:1). Visualized by charring with 25% (w/w) sodium bisulfate containing 3% (w/w) sulfuric acid.

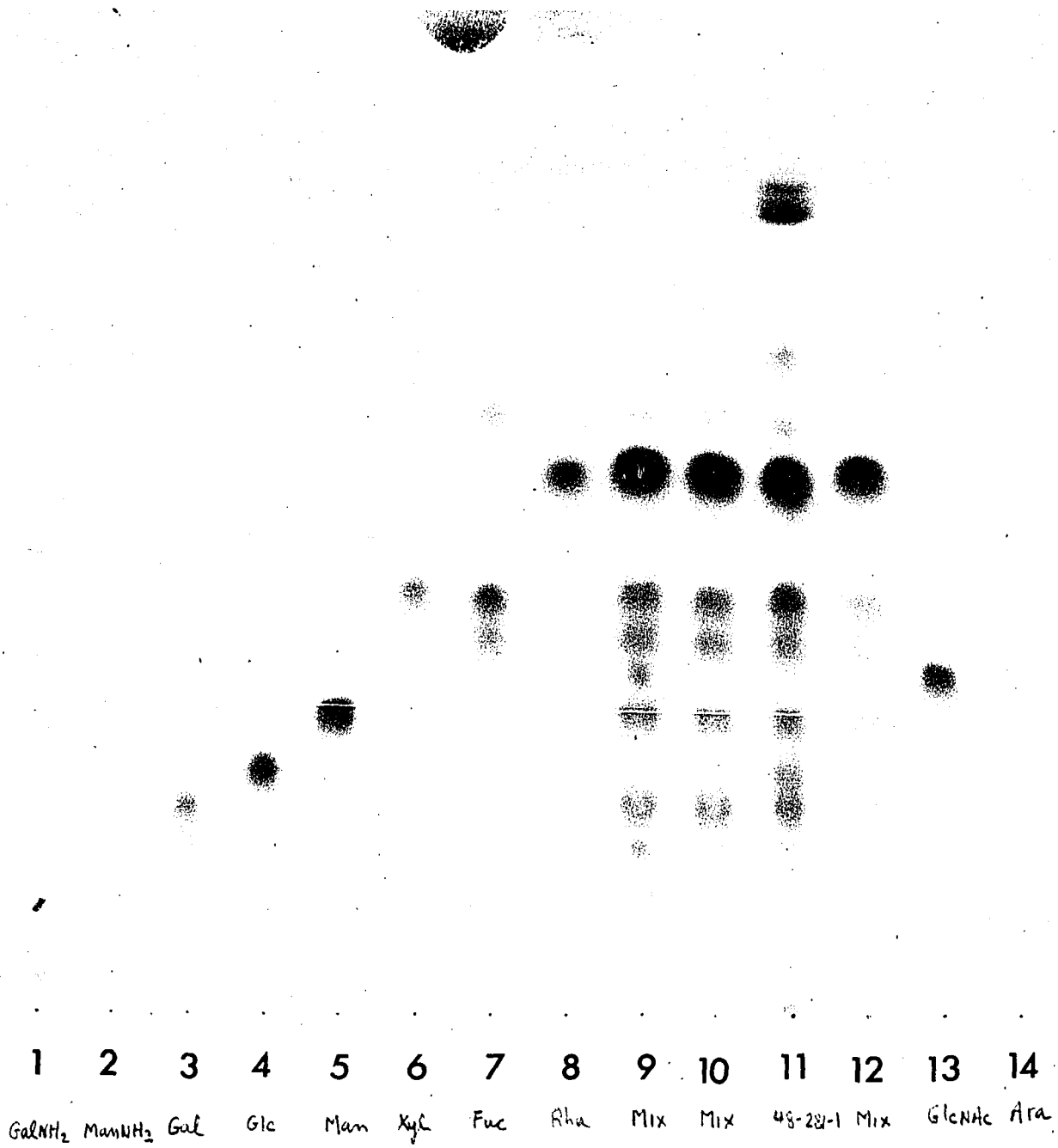

1 2 3 4 5 6 7 8 9 10 11 12 13 14
GalNH2 ManNH2 Gal Glc Man Xyl Fuc Rha Mix Mix 48-28-1 Mix GlcNAc Ara

Fig. 2

Figure 3

Thin layer chromatogram of methyl glycosides of whole flagella, digitonin and authentic sugars. Lane 1, methyl-L-fucoside and methyl-D-mannoside; 2, methyl-D-galactoside and methyl-L-rhamnoside; 3, methyl-D-glucoside and methyl-D-xyloside; 4, combination of above six sugars; 5, lipid-extracted whole flagella; 6, combination of lanes 4 and 5; 7, combination of lanes 8 and 9; 8, digitonin; 9, combination of methyl-D-galactoside, methyl-D-glucoside and methyl-D-mannoside. The plate was developed by one ascending run in benzene: ethanol: water: concentrated ammonia (100:100:15:1). Visualized by charring with 25% (w/w) sodium bisulfate containing 3% (w/w) sulfuric acid. A relatively nonpolar unknown sugar was labeled A in the thin layer chromatogram.

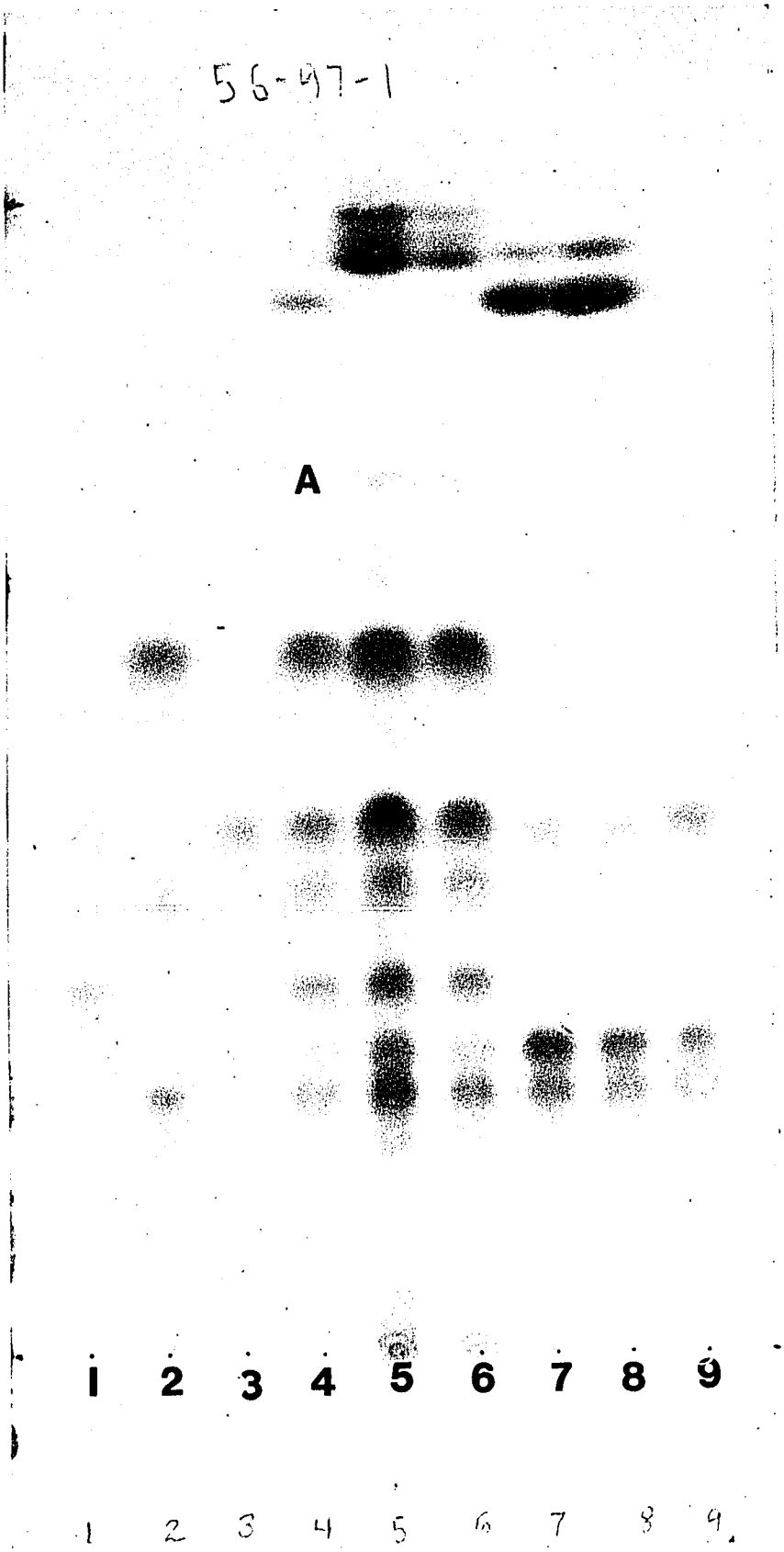


Fig.3

Similarly, the neutral and amino sugar composition of the whole flagella was checked by tlc. Lane 5 was spotted with 1.24 milligrams of lipid-extracted whole flagella. Adjacent to lane 5, an equivalent combination of the sugars in the molar proportion indicated by the glc analysis is spotted in lane 4 for comparison. The whole flagella contain the neutral sugars, amino sugars (near origin) and spot A.

Spot A, methyl rhamnoside and methyl fucoside charred differently than the other spots on the tlc plate. They first turned yellow before they darkened, whereas the other spots simply darkened. Spot A which behaved like the two methyl deoxysugars may be a deoxysugar.

The spots near the origin in the whole flagella (lane 5) are amino sugars, as shown in Figure 2. The relative ratio of neutral sugar to amino sugars of whole flagella, 15% (w/w) vs 0.4% (w/w), is in agreement with the pattern on the tlc table.

DISCUSSION

Carbohydrate Analysis - Spectrophotometric assays, gas liquid chromatography (glc) and chromatography on an absorbant are three commonly used methods for the determination of the carbohydrate composition of glycoconjugates. The carbohydrate composition is generally investigated after acid-catalyzed cleavage of the glycosidic linkages. Mild acid hydrolysis or methanolysis are relatively nondestructive procedures for liberating monosaccharides or methyl glycosides.

Levvy, et. al. (90), found that the recovery of neutral sugars and hexosamines varied with the conditions of the hydrolysis. Monosaccharide mixtures, disaccharides and ovalbumin, a glycoprotein, were hydrolyzed in either 2 M HCl at 100° for 3 hrs or 0.5 M HCl at 100° for 16 hrs. The neutral sugars and hexosamines produced were assayed colorimetrically and by glc. The recovery of neutral sugars varied from 82-94% and glucosamine from 86-98%. Neutral sugars are more labile in aqueous acid than hexosamines. These mild conditions were sufficient for the quantitative hydrolysis of di-N-acetylglucosamine units found in the sugar-containing portion of ovalbumin (92) into glucosamine and N-acetylglucosamine. (Di-N-acetylglucosamine was found in polymeric form in the extracellular microfibrils (lorica) of O. danica (45). It was clearly advantageous to remove the acid at the termination of the hydrolysis. This was done either by neutralizing the acid or removing it as an azeotrope (3N hydrochloric acid) under vacuum.

Methanolysis (with refluxing methanolic HCl) of glycoconjugates liberates the free methyl glycosides. Levvy, et. al. (90), found that methanolysis of ovalbumin quantitatively released the neutral sugars. In comparing the work of these investigators to that of Chambers and Clamp (91), one concludes that methanolysis will cleave 71% (90) to 92% (91) of the di-N-acetylglucosamine units in a known glycoprotein (as well as in other standards) into monosaccharide units. However, under these conditions the N-acetylglucosamine underwent extensive deacetylation (90-100%) (91)and the product was glucosamine, which was resistant to methylation at the hemiacetal group. Using this procedure, the hydrochloric acid is neutralized at the termination of the methanolysis with silver carbonate.

Hydrochloric acid hydrolysis (4N; heated at 100° for 5 hrs) was used in the present study to prepare monosaccharides for the quantitative determination of the total neutral sugars (83), hexoses (86) and 6-deoxyhexoses (87) by colorimetric assays. The hexosamine composition was determined by both colorimetric assay (84) and chromatography on an amino acid analyzer (88).

Colorimetric assays of samples are expressed as equivalent weight of a standard. The anthrone assay (83) is usually expressed as equivalent weights of glucose but this is a poor standard for the estimation of a mixture of sugars. Glucose, mannose, galactose, pentoses and 6-deoxyhexoses each react with anthrone. Each has a different molar extinction coefficient (and often a different absorbance maximum). Other colorimetric assays (for hexoses (86),

6-deoxyhexoses (87) and hexosamines (84) are similarly expressed as equivalent weight of a standard. However, the molar extinction coefficients differ for each of the reacting sugars in each assay. N-Acetylgalactosamine has a color intensity with the Ehrlich's reagent which is 35% of the value for N-acetylglucosamine (84). Both products have the same absorbance maximum. Thus, there is no way of sorting out which acetamidohexoses are present in the sample by this colorimetric assay. Chromatography on an amino acid analyzer, however, gives quantitative data about each separated hexosamine (88).

The principle of the three neutral sugar colorimetric assays (83, 86 and 87) is a condensation between a nucleophile (anthrone or cysteine) and furfural derivatives of the monosaccharides. The furfural derivatives are created by the addition to the monosaccharides of concentrated sulfuric acid, a powerful dehydrating agent. There was an unknown in the O. danica flagella fractions and cell bodies which reacted with the sulfuric acid and gave a dark colloidal solution. Neither the unknown nor its colored product were extracted from the aqueous samples by organic solvents. The unknown, also, did not adsorb to a mixed-bed ion-exchange resin column or to a mixture of activated charcoal-silica gel (2:1 w/w). The weight percent of total neutral sugars, hexoses and 6-deoxyhexoses in the flagella fractions could not be determined colorimetrically.

The hexosamine content of the membrane was determined colorimetrically to be 2.2% (w/w) galactosamine. This is equivalent

to 0.7% glucosamine because of the three-fold difference in the molar extinction coefficients of the colored products of the two hexosamines with the Ehrlich reagent. Only glucosamine (0.3% w/w) and no galactosamine was found utilizing the amino acid analyzer on the hydrolysates of the membrane. The amino acid analyzer identifies the hexosamines by their mobilities relative to amino-caproic acid, which is co-injected with the sample. The amount of hexosamine in the sample is determined by its ninhydrin response relative to the color intensity obtained by the known amount of added aminocaproic acid (88). There is no explanation for the difference (0.7% vs. 0.3%) in glucosamine content by the two different analytic methods.

Gas liquid chromatography was used in this study for the identification of the neutral sugars in the different flagellar fractions. The molar percent of each sugar and the weight percent of the total neutral sugars were obtained by glc. Methanolysis of many biological samples and analysis of the methyl glycosides by glc has been found to be quantitative (90, 91).

Each sugar generally chromatographs as several peaks due to the formation of pyranosides and furanosides as well as α- and β- methyl glycosides. The relative ratios of each of these peaks are constant for each sugar and are defined by the relative stability of each form under the preparative conditions. The relative amounts of each form in addition to the relative retention times of each form can therefore be used for characterization (77, 78) of each sugar. Additionally, standards were co-

chromatographed. The only sugar which was not previously studied was rhamnose. Rhamnose has only one peak on the glc. Its assignment was further verified by thin layer chromatography in several chromatographic systems (80). In some of these systems its relative position changed with respect to other known sugars.

The molar percent of the different sugars in each flagella fraction (Table III) was determined by the relative areas under the peaks. The weight percent of total neutral sugars in each fraction (Table IV) was estimated on a glc which was calibrated with known quantities of hexatrimethylsilyl myo-inositol. The area of the sugar peaks were calibrated relative to the response of the inositol derivative (Figure 1).

The methyl glycosides were also investigated on tlc. Each of the neutral sugars (with the exception of xylose) in the whole flagella was clearly separated on thin layer chromatography (Figures 2 and 3). The number of peaks seen on glc and the number of spots on tlc for each neutral sugar are identical.

The largest spot in the whole flagella had the same relative mobility and charring properties as the standard methyl rhamnoside. The tlc plates were sprayed with sulfuric acid and heated slowly. There were five spots in the whole flagella which charred yellow before darkening (the other spots did not have an intermediary yellow color). The five were the three major anomeric forms of methyl fucoside, the major anomeric form of methyl rhamnoside and an unknown, Spot A (Figure 3). The methyl fucoside and methyl rhamnoside standards similarly charred yellow before turning dark brown.

The Composition of the Flagella Membrane - The flagella fractions used in this study were obtained by treating the whole flagella with Tris buffer, pH 7.5, magnesium chloride and mercaptoethanol. The membrane, the mastigonemes, and the axonemes were separated from each other by differential centrifugation. The membrane and mastigoneme pellets appear by electron microscopy to have little contamination (37). The axoneme pellet contains mastigonemes.

The sugars present in the flagella membrane are rhamnose (major neutral sugar), fucose, xylose, mannose, galactose, glucose (Table III) and N-acetylglucosamine (Table V). The neutral sugars (1.0%) and N-acetylglucosamine (0.3%) are present in a molar ratio of approximately three to one. The neutral sugar composition of the membrane was obtained by glc. Several colorimetric assays for neutral sugars (each using sulfuric acid to dehydrate the neutral sugars) (83, 86 and 87) could not be done on the membrane as discussed above. The amino sugar content was determined colorimetrically (84). The only amino sugar that was found in the membrane, utilizing an amino acid analyzer (88), was glucosamine. This was consistent with the tlc of the membrane hydrolysate (Figure 2). Unfortunately glucosamine standard was not available but this substance chromatographs between mannosamine and galactosamine as does an unknown from the membrane hydrolysate.

The sugars most probably are present as branched glyconjugates with a poly di-N-acetylglucosamine (poly di-N-acetylchitobrose)

core attached to the protein via asparagine. It would not be surprising to find that the membrane glycoproteins contain a chitinous core since a major proteoglycan of extracellular microfibrils (lorica) of O. danica has a chitinous core (45).

The flagella membrane composition of O. danica has similarities to the plasma membranes of other unicellular eukaryotes (Table VI). There are relatively few isolations of plasma membranes of unicellular eukaryotes. Furthermore, some of these isolations were not analyzed for their sugar content and are not applicable for direct comparison to the present work (e.g.: cilia of Tetrahymena (93) and the plasma membrane of D. discoidium (94).

The percent neutral sugar of the plasma membrane of Nitzschia alba, a diatom, and the flagella membrane of O. danica are similar. The hexosamine content of N. alba is lower than that of O. danica.

Both N. alba and O. danica contain unusual anionic (pK < 1) lipids. The major flagella membrane lipid of O. danica are derivatives of 1, 14-docosanediol-1, 14-disulfate with zero to six chlorine atoms replacing hydrogen atoms on the otherwise saturated chains (31). N. alba has a ceramide sulfonic acid, where 70% of the fatty acids bound to the sphingosine are iso heptadecanoic and iso pentadacanoic acid (98).

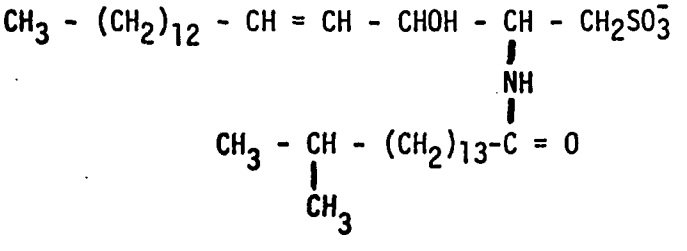


Table VI

Composition[a] of Surface Membranes of Unicellular Eukaryotes

Source of Membrane	Total Lipids	Neutral Sugars	Amino Sugars	Reference
Flagella membrane of Ochromonas danica (algae)	3.1	0.04	0.013[b]	30, Results
Pellicle of Euglena gracilis (algae)	0.12	0.21	0.006	46
Plasma membrane of Nitzchia alba (diatom)	1.0	0.07	0.002	95
Wall of Nitzschia alba ((diatom)	2.8	0.50	0.008	95
Plasma membrane of Entamoeba invadens (protozoa)	0.18	0.50	N.D.	96
Plasma membrane of Acanthamoeba castellanii (protozoa)	0.73	0.15	0.09	97

N.D. Not determined

a. The weight percent of each of the listed components is given relative to the protein content of the membrane (assigned value of 1.0).

b. Value obtained on amino acid analyzer.

The compounds like many lipids of unicellular organisms (e.g.: phosphonolipids of Tetrahymena (99) and bacteria (100) are resistant to hydrolytic enzymes, which is a desirable property in the plasma membrane lipid of an organism that does not contain a wall.

The plasma membrane of Entamoeba invadens contains a large amount of neutral sugars and has two glycoproteins and one proteoglycan (of a total of ten bands) on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (96). All of the sugars in the plasma membrane of Acanthamoeba castellanii are present in two similar low molecular weight glycoconjugates (97). The number of proteins and glycoconjugates in these membranes is small. There are similarly only five major proteins in the flagella membrane of O. danica on SDS-polyacrylamide gel electrophoresis (27).

The pellicle (a 200 nm thick region just under the plasma membrane) of Euglena gracilis has a comparable hexosamine and higher neutral sugar content than the O. danica flagella membrane (46). The neutral sugars present are rhamnose, fucose, xylose, mannose, galactose and glucose; identical to those found in the O. danica flagella. Arabinsoe, a common sugar of proteoglycans of the walls of plants and algae (e.g.: Chlamydomonas (40) is not found in the surface carbohydrates of either organism.

The Composition of the Mastigonemes - The sugars present in the mastigonemes are rhamnose (major sugar), fucose, xylose, mannose, galactose and glucose (Table III). These sugars constitute 17% of

the dry weight of the mastigonemes. The mastigonemes have less than 0.4% amino sugars. The neutral sugar composition was determined by glc.

Bouck (32) has shown that there are two types of mastigonemes in O. danica, fibrous and tubular. The tubular mastigonemes have long and short lateral filaments along their entire length (shaft) and end in a tapered basal region. The shaft and basal region are synthesized in the perinuclear continuum (membrane-bound space between the nuclear membrane and chloroplast membrane) while the filaments are added to the shaft in the Golgi complex.

The protein and glycoconjugate composition was studied. Bouck (32) found one major glycoprotein, one minor protein and several carbohydrate bands on urea-containing 7.5% acrylamide gels. Chen and Haines (27) found on 10% SDS-PAGE three glycoproteins, one carbohydrate and a sizeable fraction which did not enter the gel. The latter substance(s) stains positively with coomassie blue and periodic acid-Schiff.

The shaft and basal region of the tubular mastigonemes are probably composed of the three glycoproteins and the minor protein (seen on urea gels). Since the two types of lateral filaments are added to the shaft in the Golgi apparatus, the filaments are probably in large part or wholly composed of several proteoglycans.

The Sugar Composition of the Whole Flagella - Preparations of whole flagella contain mastigonemes, membrane and axonemes. Electron

micrographic studies have been made on Ochromonas minute (28) and O. danica (29)in which the carbohydrates have been selectively stained and only the mastigonemes and membrane appear to contain glyco-conjugates. At present there is no evidence for glyconjugates in the axonemes. Indeed the two electron micrographic staining studies of carbohydrates (28, 29) found no glycoconjugates in the interior of the flagellum. The sugar composition of the whole flagella is therefore a weighted sum of the sugar composition of the flagella membrane and that of the mastigonemes. The relative contribution of the membrane and mastigonemes to the total composition is proportional to their weight percent in the intact flagella.

The direct method for finding the weight percent of weighing the isolated membrane and mastigoneme preparations is not applicable because of losses from both fractions in the predominantly axoneme pellets. The relative volume percent of the membrane and mastigonemes can be estimated from the electron micrographs of Bouck (32) and Chen and Haines (27).

Each tubular mastigoneme consists of a hollow shaft (1.0 micron long and 18.0 nm in diameter). The thickness of the walls are 3.6 nm. Each has a basal attachment region (250 nm long and 20 nm in diameter) and approximately 400 short lateral filaments (40 nm long and 3 nm in diameter) and 40 long lateral filaments (200 nm long and 3 nm in diameter) (32). The volume of each mastigoneme is therefore 4.5 (10^{-16})cc. The thickness of the flagella membrane is 8 nm (27, 32) and the diameter of the flagella is 330 nm (32). There are seventeen tubular mastigonemes

per micron of flagella (32). The relative volume of the mastigonemes to that of the membrane is 0.92. The density of the membrane is approximately 1.13 gm/ml (27). Assuming the density of the mastigonemes to be 1.3 gm/ml the relative weight of the mastigonemes to membrane is 1.1. A ratio close to 1.0 is in agreement with SDS-gel electrophoresis protein profiles of whole flagella, mastigonemes and flagella membrane by Chen and Haines (27). The membrane and mastigonemes appear to make approximately equal contributions to the whole flagella.

The relative weight of neutral sugars of the mastigonemes to that of the membrane (Table IV) is 19 (1.1 x 0.17 / 1.0 x 0.01). Approximately 95% of the flagellar neutral sugars is therefore found in the mastigonemes and 5% is in the membrane. One would expect then, that the neutral sugar composition of the whole flagella should reflect that of the mastigonemes.

The sugar analysis of the whole flagella (Table III) showed primarily six sugars, rhamnose, fucose, xylose, mannose, galactose and glucose, in addition to amino sugars. The relative proportions of rhamnose, galactose and glucose are approximately the same for the whole flagella as they are for the mastigonemes. On the other hand, the relative mole percents of fucose, xylose and mannose are not. Perhaps the best explanation for this is that there were losses of mastigoneme hairs during the isolation of the mastigonemes. This data suggests that the hairs may be enriched in these sugars. No attempt was made to isolate mastigoneme hairs for analysis.

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