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FREE FATTY ACIDS AS A MAJOR COMPONENT OF THE CHLOROSULFOLIPID MEMBRANE OF *OCHROMONAS DANICA*

by

IRENE WINICOV

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

1985

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

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ABSTRACT

FREE FATTY ACIDS AS A MAJOR COMPONENT OF THE CHLOROSULFOLIPID MEMBRANE OF *OCHROMONAS DANICA*

by

IRENE WINICOV

Advisor: Professor Thomas H. Haines

The flagellar membrane of the phytoflagellate, *Ochromonas danica* (primarily dark-grown) had earlier been characterized by lack of phospholipids. The main polar lipids were chloro-substituted alkyl disulfates; the other principle components being free fatty acids (FFAs) and sterols. FFAs are not usually natural membrane components; they are known to uncouple proton gradients. Cell damage or solvent extraction can cause their artifactual production from esterified precursors. This work is an attempt to determine whether or not the free fatty acids are indeed components of the natural membrane of *O. danica*.

If the FFAs were artifacts, they would most likely have been produced during solvent extraction (solvent-activation of lipolysis is known to occur in plant systems) or during the procedure for flagellar detachment (mechanical wounding can also be associated with lipolysis). Attempts to denature putative solvent-activated lipase(s) through exposure to boiling isopropanol or by crosslinking the flagella with glutaraldehyde prior to extraction failed to eliminate the free fatty acid fraction, nor to

significantly alter its composition. Exposure of flagella to albumin resulted in the net transfer of FFAs to the supernatant phase, showing their presence is not caused by solvent activated lipolysis. Finally, levels of labelled free fatty acids failed to rise as a function of time after deflagellation in cells grown in the presence of [10-^{14}C]-oleic acid. Acid hydrolysis of the total labelled lipid at elevated temperature increased the percentage of counts occurring as unesterified fatty acids (from 2.6% to 64.8%). This, taken together with a corresponding loss of the more polar labelled material (66.8% to 8.2%) indicates that some esterified lipids were present, but probably not broken down during the isolation procedure.

Free fatty acids have also been reported in *O. danica's* extracellular vesicles. Indeed, liposomes can be formed using sulfolipid, sterol and free fatty acids as membrane components. The surface charge density of the flagellar membrane at low and high pH is consistent with the presence of free fatty acids in an alkyl sulfate membrane. Thus, it seems that unesterified fatty acids are a component of the chlorosulfolipid membrane of *Ochromonas danica*.

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TABLE OF CONTENTS

Page

Abstract.......... iii

Acknowledgements.......... v

Contents.......... vi

List of Tables.......... viii

List of Figures.......... ix

List of Abbreviations.......... x

Introduction.......... 1

The Lipids of Ochromonas danica.......... 3
Free Fatty Acids and Membranes.......... 7
Chlorosulfolipids, Alkyl sulfates, and Sterol sulfates as Membrane Components.......... 10
Deleterius Effects of Free Fatty Acids.......... 13
Artifactual Production of FFAs during Lipolytic Release of Free Fatty Acids.......... 14
Approaches to Establishing the Presence of Free Fatty Acids in the Natural Membrane.......... 18

Experimental Procedures.......... 22

Chemicals.......... 22
Cultures.......... 22
Harvest.......... 23
Flagella Detachment and Isolation.......... 23
Glutaraldehyde Fixation of Flagella.......... 24
Flagellar Membrane Isolation.......... 24
Extraction of Lipids.......... 25

Page

Thin Layer Chromatography.....27
Isolation of Lipids by Preparative Chromatography.....27
Gas Liquid Chromatography.....28
Adsorption of Free Fatty Acids using Albumin.....29
Time Study of Free Fatty Acids after Flagellar Detachment.....30
Autoradiography.....32
Isolation of Radioactive Lipids.....32
Acid Hydrolysis of Lipids.....33
Liquid Scintillation Counting.....33
Surface pH Study of the Flagellar Membrane.....34
Protein Assay.....35
Azure A Assay for Sulfolipid.....35

Results.....37

Lipase Inhibition.....36
Albumin Adsorption of Flagellar Free Fatty Acids.....41
Flagellar Detachment Study.....43
Surface pH Study.....60

Discussion.....68

List of References.....85

LIST OF TABLES

Page

1. Comparison of Extraction Procedures Designed to Inhibit Putative Lipase Activity: Quantities of Isolated Free Fatty Acids........................38

2. Albumin Adsorption of Free Fatty Acids from Flagella..................................42

3. Distribution of Counts among Cell Fractions after Growth on [10-^{14}C]-Oleic Acid. Initial Cell Harvest..46

4. Distribution of Counts among Cell Fractions after Growth on Labelled Oleic Acid: With and Without (±) Deflagellation......................46

5. Composition of Major Labelled Lipids appearing on Autoradiogram, as % Total Dpm. (<2 hours exposure)..53

6. Composition of Major Labelled Lipids appearing on Autoradiogram, as % Total Dpm. (Three days growth)..55

7. Counts appearing as Free Fatty Acid in the Supernatant of Cells With and Without (±) Deflagellation.(18 hours growth)...............................60

8. Surface pH Measurements Using Alkylated Coumarin Indicators...............67

LIST OF FIGURES

Page

1. The Chlorosulfolipids of *O. dancia*..........6
2. Flagellar Membrane Lipid Composition..........8
3. Free Fatty Acids Have a Unique Composition..........40
4. Autoradiogram of Lipids from *O. danica* After < 2 Hours and 3 Days Growth in the Presence of [10-^{14}C]-Oleic Acid..........50
4. Autoradiogram of Lipids from *O. danica* Supernatant After 18 Hours Growth in the Presence of [10-^{14}C]-Oleic Acid..........52
6. Surface pH Probes..........64
7. Dissociation of Coumarin Indicators as a Function of Bulk pH: Free vs. Hydrophobically Bound to Neutral or Anionic Surfaces..........65

Abbreviations

BSA	Bovine serum albumin
ECV	Extracellular vesicle
EDTA	Ethylenediamine tetraacetic acid
FFA	Free fatty acid
GLC	Gas liquid chromatography
HSA	Human serum albumin
NMR	Nuclear magnetic resonance
PG	Phosphatidyl glycerol
PI	Phosphatidyl inositol
SDS	Sodium dodecyl sulfate
SOS	Sodium oleyl sulfate
TEM	10 mM Tris, 1 mM EDTA; 1 mM ß-Mercaptoethanol, pH 7.5
THAGE	1,2-Diacylglycerol-(3)-O-4′-(N,N,N-trimethyl)-homoserine
TLC	Thin layer chromatography

INTRODUCTION

Lipids are classified according to their chemical characteristics as either polar or non-polar. The headgroups of the polar lipids contain phosphate, glycoside, and/or sulfate. At physiological pH, polar lipids are either negatively charged ("acidic"): phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), or zwitterionic: phosphatidylcholine (PC), phosphatidylethanolamine (PE), etc. Polar lipids are usually found esterified to either glycerol (two fatty acyl chains), or to sphingosine (one fatty acyl chain). Lipids containing one polar component with two long chains have been shown to spontaneously form bilayer structures (liposomes) in aqueous solution such that the polar portion ("headgroup") is oriented towards the aqueous phase, and the nonpolar hydrocarbon region is "sandwiched" inside. The absence of one fatty acid chain (a lysolipid) produces a detergent-like molecule which neither forms nor stabilizes bilayers. Indeed, it disrupts them.

The membrane nonpolar lipids are not ionic. They may include hydrocarbons, sterols, sterol esters, waxes, isoprenes, and acylglycerides. Most tri-, di- and mono-glycerides are normally components of storage lipids, and are not presumed to be natural membrane components. Fatty acids are rarely found as components of membranes. Early reports of free fatty acids in membranes were later found to be the result of lipase activity. The nonpolar lipids (e.g. sterols) do not spontaneously form bilayers in aqueous solution.

Free fatty acids and detergents such as sodium dodecyl sulfate (SDS) are structurally similar in that they contain a single hydrophobic chain and

a charged, hydrophilic region (ionized sulfate or carboxylate headgroup). In aqueous solutions, they form micelles, which are complexes of molecules oriented so that their polar moiety faces the aqueous solution, and the hydrophobic chains are sequestered within the complex. They differ from liposomes or vesicles in that micelles lack an entrapped aqueous compartment.

In view of the strong tendency for detergents to form micelles which are disruptive of membrane structure, it was surprising when free fatty acids were found to form membrane-like structures. Gebicki and Hicks (1973) prepared liposomes (containing a trapped aqueous comparment) from unsaturated fatty acids alone. Hargreaves and Deamer (1976) showed that unsaturation was not a requirement for preparation of fatty acid liposomes. They would form from any fatty acid provided they were above their transition temperature (T_m). Thus, short chain fatty acids (C14) form liposomes at room temperature, and long chain saturated fatty acids (C18) at elevated temperatures. These liposomes were stable within a pH range of 7 to 9.6. These authors also realized that the protonation state of the headgroups (ie, the charge density) influences the stability of these and other detergent liposomes. In the pH range of 7 to 9.6, the carboxylates at the liposome surface are near their pK[1], i.e., there are equal amounts of protonated and unprotonated carboxyls. Hargreaves and Deamer (1978) were also able to form liposomes from alkyl sulfates such as sodium dodecyl sulfate (SDS) provided that neutral "spacer" molecules such as

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[1]An explanation for the unusually high pK_a values commonly observed for long chain carboxylates will be found in "Discussion".

long chain alcohols or sterols were present.

The Lipids of *Ochromonas danica*

O. danica is a fresh water phytoflagellate which grows optimally at pH 4.5 and possesses unusual, detergent-like membrane lipids. Unlike many other microbes, *O. danica* lacks a visible cell wall when observed under electron microscopy. Therefore, protection of the membrane from lipolytic enzyme attack by predators must be accomplished through its membrane lipids. The absence of a cell wall also raises a question as to how this freshwater organism resists osmotic swelling. This would presumably require an unusually stable membrane. Two other eukaryotic organisms also known to lack a cell wall (*Tetrahymena pyriformis*, and *Paramecium tetraurelia*) contain highly unusual lipids in their (ciliary) membranes (Rosenberg 1974; Kennedy and Thompson 1970; Nozawa and Thompson 1971; Rhoads and Kaneshiro 1979). These lipids, the phosphonolipids, have been shown to be more stable against attack by exogenous phospholipases than other phospholipids, and may even be inhibitory to these enzymes (Hori and Nozawa 1982; Rosenthal and Pousada 1968; Rosenthal and Ham 1970). Most of the phosphonolipids of *Tetrahymena* are concentrated in the external surface membranes: the cilia and pellicle. In the internal membranes (mitochondrial, nuclear envelope, and microsomes) the more "usual" lipids (PC, PE) predominate (Nozawa and Thompson 1971). We will see that the some of the "unusual" lipids in *O. danica* may similarly be concentrated in the surface membranes.

Whole Cell Extracts. Lipid extracts of dark-grown *O. danica* (no chloroplasts) have an unusually low content of phospholipid in proportion to the amounts of sulfur- and nitrogen-containing lipids (0.24µmol[P], 15.6µmol[S], and 3.77 µmol[N] per 10^9 cells, respectively (Brown and Elovson 1974; Chen et al. 1976). Elovson and Brown had reported a sulfolipid concentration of 5.88µmol per 10^9 cells, but the actual quantity is higher since the extraction method[2] used by these authors selectively excludes many of the very polar sulfur-containing lipids. The data of Chen et al. (1976) showed sulfolipid to be 53.8% of the total lipids on a molar basis. Both groups measured the quantity of THAGE, a lipid to be described below, (9.6 mol%, Chen et al. 1976; 2.78µmol per 10^9 cells, Elovson and Brown 1974). Thus, Elvoson and Brown's data can be adjusted to reflect the total sulfolipid: (53.8/9.6)(2.78 µmol THAGE per 10^9 cells) = 15.6 µmol [S] per 10^9 cells.

The phospholipid composition of *O. danica* seems to be characteristic of mitochondrial lipids, *i.e*, PE and PC were found in dark grown cells whereas PS and PI were absent in the extracts.

The sulfolipids of *O. danica* are present as alkyl disulfates and their chlorinated derivatives (shown in Figure 1). These are primarily 1,14-docosanediol-1,14- disulfate and its chlorinated analogues, with a smaller quantity (about 5% of the total) of 1,15-tetracosanediol-1,15-disulfate and its chlorinated derivatives. (Elovson and Vagelos 1969; Haines et al. 1969; Elovson and Vagelos 1970; Haines 1965; Mayers and Haines 1967;

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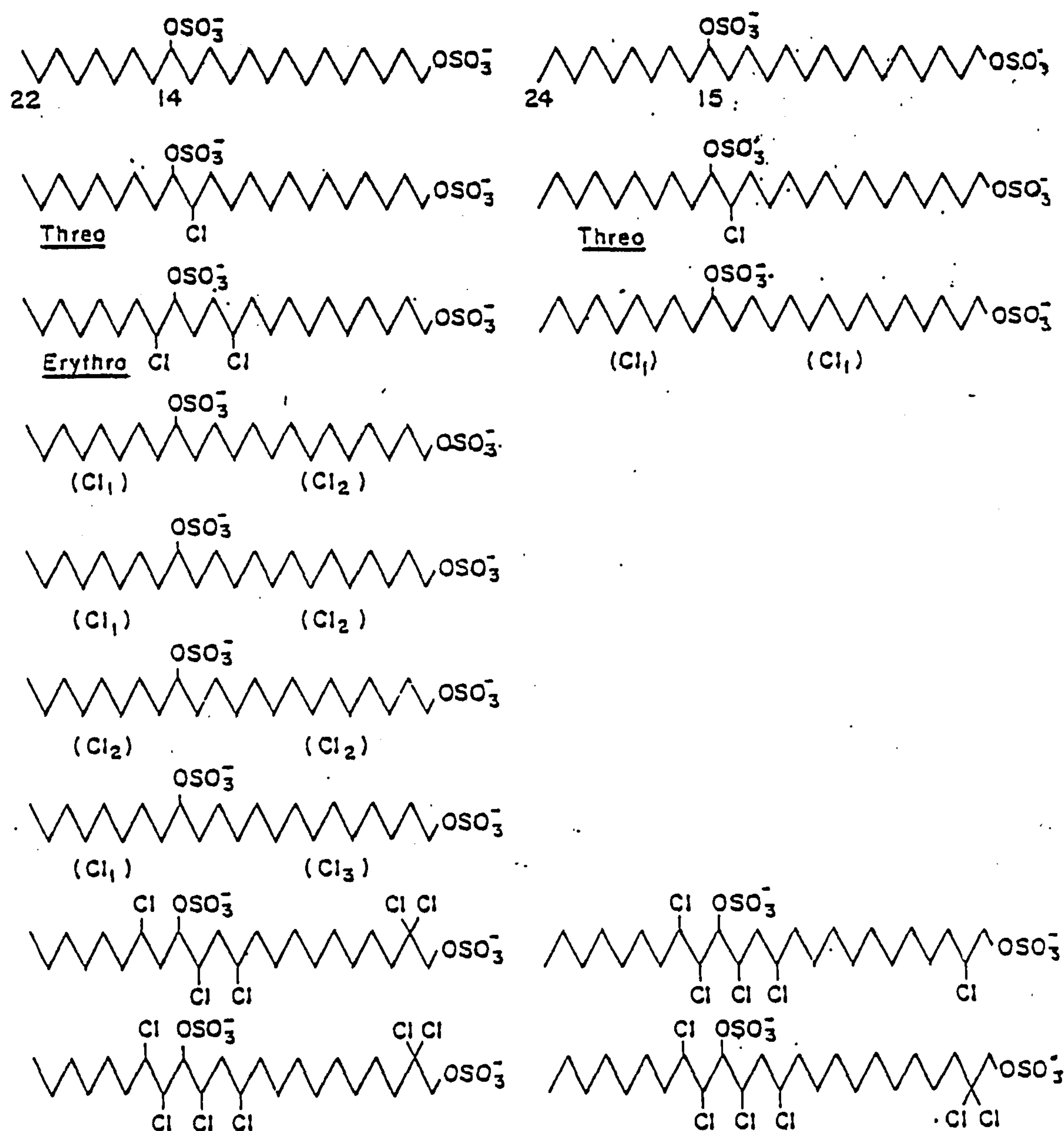
[2]essentially that of Bligh and Dyer (1959), modified by the inclusion of EDTA in the aqueous phase. Only the lower phase lipids were examined; however, most of *O. danica's* sulfolipids partition into the upper phase.

FIGURE 1



Structures of the chlorosulfolipids of *O. danica*; from Haines (1973).

Mayers et al. 1969; Haines 1971; Haines 1973).

Smaller quantities of esterified lipids are present. One of these, representing nearly 10% of the total lipids, has the structure 1(3),2-diacylglyceryl-(3)-O-4'-(N,N,N-trimethyl)homoserine (THAGE), (Brown and Elovson 1974). Free fatty acids (22% of the total lipids), including arachidonate (11%) and other polyunsaturates, were found in high quantities (Haines et al. 1962). Unesterified sterols (7% of the total; Chen et al. 1976) of the algal type (Gershengorn et al. 1968) were found *i.e.*, with a branch at C_{24} of the S configuration; higher plants and yeast have the R configuration for this branch.

Flagellar Membrane Lipid Extracts. Because of the difficulty in obtaining pure preparations of a single membrane such as the plasma membrane from *O. danica* (Elovson and Vagelos 1969), the flagella (which can be detached) were first isolated. A purified preparation of its membrane could then be obtained (Chen et al. 1976). Membranous extra-cellular vesicles (ECVs) are released by the organism into its growth medium (Aaronson 1971) and originate from both the plasma and flagellar membrane. The lipid composition of the ECVs and flagellar membrane are similar (El Maraghy 1982). Considering the physical continuity of the flagellar and plasma membranes, and the similar composition of the the ECVs (arising from both membranes), the membrane lipid composition of the flagella would seem to be a model for that of the plasma membrane.

Elemental analysis showed phospholipid to be entirely absent from the flagellar membrane (Chen et al. 1976) and from ECVs (El Maraghy 1982). Both membrane preparations contain three classes of lipids representing

over 90 mol% of their total lipids: the alkyl disulfates, free fatty acids and sterols. The alkyl disulfates comprise 71% of the lipids (an increase over the percentage of this lipid in whole cell extracts). More than 12% of the lipids were isolated as unesterified fatty acids, and close to 10% were sterols (Chen et al. 1976; El Maraghy 1982). The composition of the flagellar membrane lipids is depicted in Figure 2.

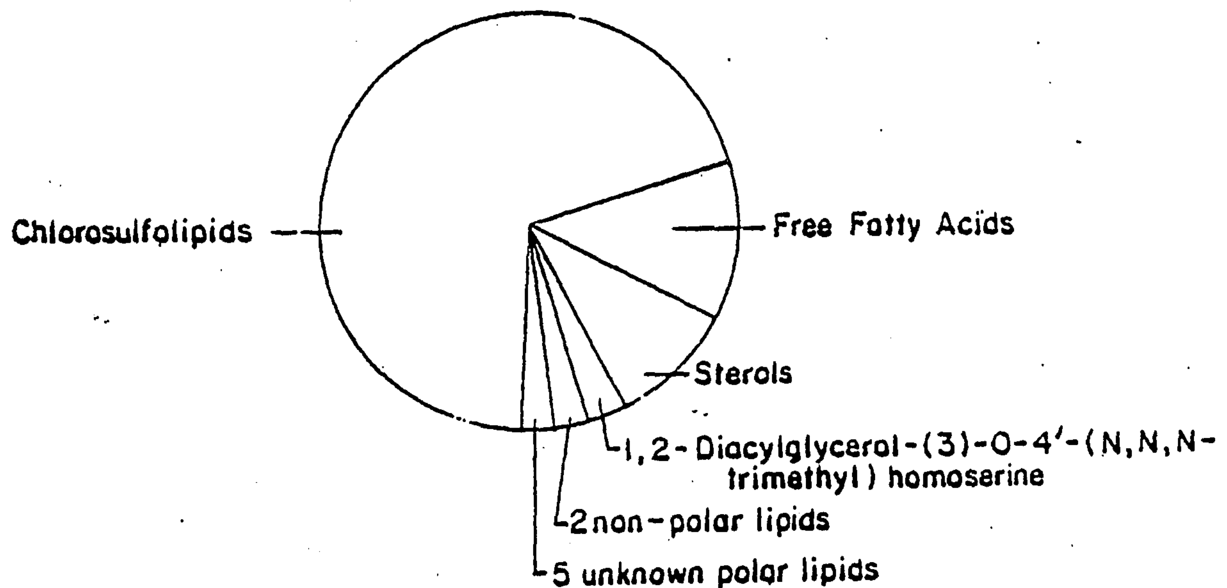
Only very low quantities of esterified fatty acids were associated with the isolated membrane. THAGE represented 1.3% of the total lipids (a lower proportion than found in whole cell extracts), and less than 1.5% (the sum) of the other unknown polar lipids were esterified. This suggests that, as in *Tetrahymena*, a highly unusual class of lipid (chlorosulfolipid) may be concentrated in an "external" membrane, with more "typical" lipids found in internal membranes. Although the structure of THAGE is unusual for most membranes, it more closely resembles the structure of phosphatidylcholine (a "classical" membrane lipid) than do the chlorosulfolipids. THAGE seems to be less concentrated in the flagella than in other organelles, since the percent composition is higher in whole cell extracts than in isolated flagella.

Free Fatty Acids and Membranes

Free Fatty Acids as Membrane Components. Most fatty acids found in membranes are esterified to either glycerol, cholesterol, or sphingosine. Fatty acids have also been found covalently bound to certain membrane proteins (Schlesinger 1981). Only very low amounts of free fatty acids (less than 1%) are normally found in extracts of typical

FIGURE 2

LIPID COMPOSITION OF THE FLAGELLAR MEMBRANE OF *Ochromonas danica*



Composition (mol%) of the flagellar membrane lipids; re-drawn from data of Chen et al. (1976).

phospholipid membranes. These are generally attributed to chemical or enzymatic degradation during isolation. FFAs are often liberated after wounding or other damage to cells, and are sometimes the product of solvent-activated lipases. Furthermore, in large quantities they have been shown to disrupt many membrane functions. These possibilities will be more fully discussed below. However, elevated levels of free fatty acids have occasionally been reported in whole cell or even membrane extracts. This raises the *possibility* that FFAs may be membrane components.

Phospholipid membranes that contain larger than typical quantities of free fatty acids include the retinal rod outer segment membranes (Miljanich et al.[3]) and epidermal cell membranes (Gray and Yardley 1975). Neither of these membranes contain important transport activities. The absorption of free fatty acids in the mammalian intestine is coupled with bile acids. It would therefore not be surprising to find free fatty acids in intestinal epithelial cell membranes, and indeed they are present (Kawai et al. 1974).

Free Fatty Acids and Sulfolipids. Lipid and/or membrane extracts containing alkyl sulfate frequently also contain free fatty acids. For example, both types of compounds are reported in brown algae (Laur and Liem 1970; Liem and Laur 1974; 1976) and in the phytoflagellate, *O. danica* (Chen et al. 1976). Likewise, lipid extracts rich in sterol sulfates have been found to contain relatively high quantities of free fatty acids. Examples include *Nitzchia alba* (Anderson et al. 1978), epidermal cells (Gray and Yardley 1975), intestinal cell membranes (Kawai et al. 1974), and sperm (Keenen et al. 1972; Bleau and Van den Heuvel 1974; Levine et al. 1976).

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[3]unpublished work, cited by Dratz (1983)

Chlorosulfolipids, Alkyl sulfates, and Sterol sulfates as Membrane Components.

Mercer and Davies (1979) found chlorinated alkyl sulfates to be present in over 30 species of fresh water algae but absent from 8 types of marine algae. Chlorosulfolipds usually represent only a small portion (less than 2%) of an organisms' total lipids, but in *O. danica*, they represent more than 14% of the total cellular lipids. These authors had previously noted the presence of alkyl sulfates in three Chlorophytes (green algae), two Xanthophytes and two Cyanophytes as well as in one other Chrysophyte besides *O. danica* (Mercer and Davies 1974,1975). Liem and Laur (1974;1976) characterized alkyl disulfates different from those in *O. danica* in three species of brown algae (Fucacea) from the Brittainy coast: *Fucus canaliculata* (L.), *F. vesiculosus* (L.), and *F. serratios* (L.). These alkyl sulfates are: 1,18-tricosanediol-1,18-disulfate; n-tricosane-diol sulfate; 1,6-octadecanediol-1,6-disulfate; and 10-eicosene-1,8-diol-1,8-disulfate. These authors also reported the presence of significant levels of free fatty acids in the same organisms (Laur and Liem 1970).

Halide containing lipids (endogenously synthesized) may also be widespread (reviewed in Addison 1982). Small quantities of acid labile bromo-lipids which are closely associated with fatty acids have been reported in certain marine organisms. Chloro-compounds have also been found in even higher quanities in many marine organisms, and many are stable to strong acids. A jellyfish containing fatty acid chlorohydrins (e.g., 9-chloro-10-hydroxy-palmitic and stearic acid) has been reported (White

and Hager 1977).

Sulfolipids (but not necessarily alkyl sulfates) may generally be associated with acidic membranes (Haines 1984). This is of great interest when one notes that the membranes of the earliest organisms, the archaebacteria (Fox et al. 1980), are acidic. This class includes the halophiles; the purple membrane of *Halobacterium cutirubrum* even contains sulfated lipids (Kushwaha et al. 1975). Indeed, all known energy transducing membranes -- mitochondrial and photosynthetic membranes in both prokaryotes and eukaryotes (Benson 197 , Wood et al. 1965) -- contain acidic lipids. In higher plants and animals, the sulfate group is more commonly found attached to a carbohydrate moiety or even to sterol rather than to an alkyl chain. A correlation between cerebroside sulfate content and Na,K^+-ATPase activity in several vertebrate tissues has been observed (Karlsson et al. 1971; 1974). Sterol sulfate found in organisms which seem to lack cerebroside sulfates may also play a role in ion transport (Pascher and Sundell 1977).

Sulfolipids are often associated with flagellar membranes (but not with cilia) and are also present in the sperm of all known species. Examples of these lipids include sulfogalactosylmonoalkylmonoacylgycerol (man) and sulfogalactosylceramide (bird and fish); they are not found in immature testis (Levine et al. 1976). These lipids are located in the plasma membrane (Klugerman and Kornblatt 1980) and presumably in the flagella as well. Sterol sulfates have also been reported in sperm membrane (Bleau and Van den Heuvel 1974).

Most photosynthetic plants contain sulfoquinovosyldiglyceride in their chloroplast membranes. Mercer and Davies (1979) noted in their survey of

freshwater and marine algae that sulfolipid in a given organism was generally present in the form of either alkylsulfate or sulfoquinovosyl diglyceride, but not both.

Sterol sulfates are present in many types of eukaryotic cells. First isolated from bovine adrenals (Drayer et al. 1964), low levels of cholesterol sulfate has been reported in human brain, erythrocytes, kidney, liver, urine, and bile (Moser et al. 1966), and in epidermal cells (Gray and Yardley 1975). The highest amounts occur in feces (*ibid.*). Physiological levels of this lipid have been shown to play a role in the erythrocyte's maintainance of shape under hypotonic stress (Bleau et al. 1974). Indeed, cholesterol sulfate has been proposed as a natural membrane component of erthyrocytes (Bleau et al. 1975). This compound is also a major constituent of intestinal brushborder membrane (Karlsson) and its appearence in feces may be due to the rapid turnover of this membrane.

Sterol sulfates have been found in invertebrates including echinoderms (Goodfellow and Goad 1983), the sea star, *Asterias rubens* (Bjorkman et al. 1972), and a sea sponge, *Toxidocia zumi* (Nakatsu et al. 1982). They have also been found in the non-photosynthetic diatom, *Nitzchia alba* (Anderson et al. 1978). The high levels found in *A. rubens* (1.3 mg/g dry tissue, Bjorkman et al. 1972) have led to the suggestion that it is a membrane component in this organism. The sterol sulfates of *T. zumi* are noteworthy in that they contain both a sulfate and a carboxylate group on the same sterol ring. They are released into the medium, and were suggested to function as antimicrobial agents (Nakatsu et al. 1982).

Deleterius Effects of Free Fatty Acids

Disruption of Proton Gradients. Unesterified fatty acids have been shown to equilibrate proton gradients in several systems, including mitochondria (Racker 1979) and chromaffin granules (Husebye and Flatmark 1984); they should thus be injurious to prokaryotic and other membranes. This may form the basis of their anti-microbial effect. Sub-micromolar concentrations of free fatty acids regulate a protein responsible for uncoupling respiration in brown adipose tissue during thermogenesis (Heaton and Nicholls 1976; Rial et al. 1983). The presence of this protein increases the mitochondria's normal sensitivity to FFAs as uncouplers by at least one order of magnitude.

Other Effects. Free fatty acids are known to form one of the defenses of plants against infection, and can be applied topically (to most plants) to control insects. They inhibit the growth of certain protozoa (Omerod and Venkatesan 1982) and bacteria (Davis and Dubos 1947). Massive thrombosis has been shown to occur shortly after infusion of free fatty acids into the bloodstream (Conner et al. 1963) and they are considered to be a major factor in heart and skeletal muscle lesions arising from ischemia, stress damage, and hypoxia. This is presumably why fatty acids are not found free in the bloodstream of mammals, but are instead combined with albumin and other proteins for transport. The complexing of free fatty acids has also been proposed as a molecular mechanism responsible for membrane stabilization by vitamin E (Erin et al. 1984). Although free fatty acids may be found in a membrane preparation one may

not be confident that they are present *in vivo* as an important component of the membrane.

Artifactual Production of FFAs during Lipolytic Release of Free Fatty Acids.

Flagellar detachment or lipid isolation should be seen in the context of systems in which lipolytic release of free fatty acids is known to occur. Particularly well documented examples of enzymatic lipid degradation causing widespread release of FFAs include: the mobilization of fats during seed germination, the response of higher plants to wounding (from mechanical or microbial damage), and more generally (and on a smaller net scale), hormone- or neurotransmitter binding events causing stimulation of phospholipases in mammalian cells. Free fatty acids are presumably formed during the routine turnover of phospholipids. Although many of these cases of lipolysis occur in "higher" plants or animals--cells showing a greater degree of specialized function than would be expected in *O. danica* --they give some idea of the extremes of possibilities. Let us examine the best characterized of these systems:

Mobilization of Fats in Seed Germination. Mammalian and many other cells growing in culture have a tendency to accumulate lipid droplets under a number of circumstances, including growth in a lipid-rich medium (containing free fatty acids), exposure to various hormones, or lowering of the pH (Spector et al. 1981). Triglycerides (Hitchcock 1975) and sometimes, other acyl lipids, are often stored in these large deposits. They are found in high quantities per cell in plant seeds, and in white adipose tissue in mammals. Lipid bodies also occur in lesser number in a variety of plant

leaves and other cells, including *O. danica*. Here they probably represent short-term energy storage.

In mature seeds, these storage lipids are rapidly mobilized in response to hydration during seed germination. Triglycerides are rapidly degraded by lipases located in the membrane of the lipid bodies (Moreau et al. 1980). Degradation is closely associated with ß-oxidation, the glyoxylate cycle, and gluconeogenesis. Most glyoxysomes have alkaline lipases associated with their membranes, although a lipase (pH optimum at 4.2) from castor bean was shown to be associated with the lipid body membrane (Ory et al. 1968).

Free Fatty Acid Release in Response to Mechanical or other Wounding. Mechanical wounding of the storage organs of some higher plants can cause rapid and wide-scale enzymatic degradation of acyl lipids. The effect is most pronounced in potato tubers. Within seconds of slicing potato tubers at 0 °C, a 20% loss of acyl lipids was seen. Within minutes, 40% of membrane lipids were reported to have been degraded (Hasson and Laties 1976). This rapid degradation has been linked to the presence of a highly active lipid acyl hydrolase, or hydrolases (Galliard 1978). The enzyme activity is found in the soluble fraction of the cells, but probably originates in lysosomes or vacuoles which are ruptured during homogenization. Lipase(s) can catalyze the breakdown of 5-50 µmoles galactolipid/min/gram fresh tissue (Galliard 1978), and is also active against a wide range of polar lipids. The degradation process is autocatalytic, since the enzyme is stimulated by free fatty acids, especialy linolenic acid. The presence of relatively high quantities of added BSA (0.1-1%) causes a decrease in the rate of enzyme activity. High concentrations of BSA are often added to

protect mitochondria from the deleterius effects of acyl hydrolase-liberated free fatty acids during their isolation. However, the response to mechanical wounding varies in severity from species to species, with the potato tuber representing the highest reported extreme; spinach leaves show much lower values. The activity of lipolytic enzymes will likewise be expected to vary among species.

The wounding response in potato tubers is also associated with high rates of respiration. The free fatty acids generated by acyl hydrolase are further metabolized by α-oxidation, and by the lipoxygenase system. In some cases, the loss of acyl lipids is much greater than would be expected based on the increase in free fatty acids, presumably due to rapid metabolism of the free fatty acids (see Galliard 1978).

Extensive autolysis has also been reported during homogenization of protozoan (Thompson 1969), plant (Galliard 1978), and mammalian cells (Kramer and Hulan 1978).

Free Fatty Acid Release in Receptor Binding Events. The appearance of elevated quantities of free fatty acids due to phospholipase activation is common to a number of hormone (insulin, thrombin, peptide) and neurotransmittor (adrenergic, muscarinic, cholinergic) receptor binding events (McPhail et al. 1984). A phosphoinositol-specific phospholipase C releases both diacylglyceride and the phosphoinositol headgroup (reviewed by Berridge and Irvine 1984). Free fatty acids are ultimately released from the diacylglyceride through the action of either a diacylglyceride lipase (Dixon and Hokin 1984), or in some systems, a phospholipase A-type enzyme (Berridge and Irvine 1984). These events are also associated with increases in the level of intracellular Ca^{2+} (Ca^{2+} regulates most membrane-bound

phospholipase A-type enzymes, Van Den Bosch 1982). Since arachidonic acid is a major component of PI, prostaglandin synthesis can also be initiated by this chain of events. Binding of norepinephrine to brown adipocytes triggers the breakdown of triglycerides through activation of a lipase by cyclic AMP-stimulated protein kinases (Skala and Knight 1977). Net release of FFA is greatly stimulated even though fatty acid oxidation is stimulated in parallel (Nicholls 1979).

Free fatty acids alone, in the absence of hormone-receptor binding events, cause many of the hormone stimulated behavioral events. It is not always clear whether the effects of free fatty acids occurs through the direct action of the fatty acid, or through further metabolites such as prostaglandins or endoperoxides. FFAs (in the absence of hormone) induce the respiratory burst in neutrophils as well as chemotaxis in mammalian lung and pleura macrophages (Lynn et al. 1981). The effect on macrophages is particularly interesting in light of the fact that these cells, like *O. danica*, are phagocytes which release extracellular free fatty acids (Lynn et al. 1981). Because of these and other effects, free fatty acids have recently been proposed as a "second messenger" (McPhail et al. 1984).

Free fatty acids (arachidonic, linolenic, linoleic and oleic acid) further increase diacylglyceride-stimulated protein kinase C activity at physiological concentrations in human neutrophils (McPhail et al. 1984). Physiological concentrations of FFAs have been shown to inhibit the conversion of Na-K ATPase from the E1 (high affinity for ATP) to E2 (high affinity for K^+) conformation (Swann 1984). The effect on the Na-K ATPase was most pronounced with polyunsaturated fatty acids (arachidonate, linolenate, etc) and may be related to changes in membrane fluidity, but is

not due to non-specific "detergent" effects or to lipid peroxidation (Swann 1984).

Although their massive accumulation may lead to cell destruction, smaller quantities of free fatty acids may perform essential functions. One characteristic of the systems mentioned above would seem to be that free fatty acids are usually rather transitory membrane components, perhaps being released to signify particular binding events. In large quantities, they are toxic. Their toxicity at high levels could be due to non-specific, detergent-like effects (disruption of lipid-protein interactions, or dissipation of proton gradients); to specific interactions with proteins; or to the action of their metabolites.

Approaches to Establishing the Presence of Free Fatty Acids in the Natural Membrane.

We have seen that free fatty acids are rarely found in phospholipid membranes in large quantities. We have also seen that they are capable of forming bilayers, both alone and in the presence of alkyl sulfates. Is the alkyl sulfate membrane of *O. danica* organized around the presence of a relatively large amount of unesterified fatty acid, or are they artifacts associated either with damage to the organism or extraction and isolation of its lipids? Our approach has been to design methods that would inhibit production of free fatty acids, or to establish that free fatty acids are present prior to the extraction of the lipids.

If the free fatty acids isolated from flagellar membrane are artifactual, they must have arisen from a larger esterified compound. But

the membrane of *O. danica* has an unusual lipid composition (sulfolipid rather than phospholipid based); it is therefore not clear what sort of compound this might be. Specific enzyme inhibitors did not seem to be the method of choice for determining whether free fatty acids are natural membrane components.

Solvent-activated lipolysis was early shown in certain plant systems. Such lipolysis had to be identified or ruled out by extracting the membrane under conditions which are known to inactivate lipases. Lipases are esterases that function in a heterogeneous environment, *e.g.* at the interface between an emulsified lipid and its aqueous phase (Brockerhoff and Jensen 1974). Extraction using solvent systems containing alcohol often eliminates this problem by inactivating many lipases (Kates 1972). However, some enzymes (such as phospholipase D from runner bean or sugar beet) are particularly stable to solvents. They have been successfully inactivated by a brief exposure to either boiling alcohol or water (Kates and Eberhardt 1957).

Another approach to eliminating the possible release of FFA by a solvent-activated lipase is to expose the membrane to albumin prior to its extraction with solvent. The finding of albumin-bound free fatty acids with the same composition as that of the natural membrane may be taken as proof that the fatty acids are not produced by a solvent-activated lipase, although it would not preclude their production at an earlier stage of the isolation procedure.

Another method which might limit lipolysis would be to fix the cells in glutaraldehye prior to extraction. The reagent would not necessarily inactivate a putative lipase, but could be expected to limit its access to

substrate by crosslinking it to the insoluble cell residue. This would reduce its potential time in contact with substrate, and should depress the quantities of (putative) lipase-generated fatty acids. Other studies of glutaraldehye-fixed cells suggested that this treatment should permit quantitative extraction of most nonpolar lipids, and a lower recovery of polar lipids (Korn and Weisman 1966).

Another approach to establishing the presence of free fatty acids in the isolated membrane is to examine the flagellar membrane surface charge density above and below the pK of the carboxylate. This is accomplished by measuring the surface pH with indicator probes at two bulk phase pHs. Alkylated derivatives of aminocoumarin and hydroxycoumarin were first introduced by Fromhertz (1973) to examine the pH at the surface of micelles. This method was further refined and integrated into a thermodynamic theory by Fernandez and Fromhertz (1977). Alkylation of the coumarin moiety anchors a portion of the molecule into the low dielectric region of the bilayer while localizing the indicator group within the headgroup region (Pohl 1976). The surface pH and thereby the charge density at the surface of a flagellar membrane preparation can be measured and compared to model membranes with and without free fatty acids.

Even if it is shown that the free fatty acids are present in the isolated membrane preparation, it could be argued that they were released from a precursor at an earlier stage in the isolation procedure. Investigations into whether or not the flagellar detachment procedure caused significant lipolysis (as in wounding of plant tissue) were also made.

It should be noted that free fatty acids are readily taken up from its medium by *O. danica* (Mooney et al. 1972); oleic acid is a major component

of the flagellar membrane FFAs (Chen et al. 1976) and is not on the direct biosynthetic pathway for sulfolipids, as are some of the saturated fatty acids (Mooney et al. 1972; Elovson 1974). Cells were cultured in the presence of ^{14}C-oleic acid and their flagella were detached. A large, time dependent increase in the quantity of free fatty acids would be observed after deflagellation if this treatment were to activate a lipase.

EXPERIMENTAL PROCEDURES

Chemicals

All chemicals were reagent grade. Tryptose Blood Agar was obtained from Difco Labs, Detroit, MI. Glutaraldehyde and SDS (sodium dodecyl sulfate, 99% pure) were from Polysciences, Warrington, PA. 2,2-Dimethoxy-propane in dry methanolic HCL (3N), and boron trifluoride in methanol were obtained from Supelco, Bellefonte, PA. Diazald (N-methyl-N-nitroso-p-toluene-sulfonamide), and Azure A (3-amino-7-diemethylaminopheno-thiazine-5-ium chloride, 81%), were from Aldrich, Milwaukee, WI. Fatty acid methyl ester standards for gas chromatography were from either Supelco or Nuchek Prep, Elysian, MN. Pentadecanoic acid was also from Nuchek prep. Human serum albumin, delipidated fraction V, was obtained from Sigma Chemical Co., St Louis, MO. [10-^{14}C]-Oleic acid was from Research Products International Corp., Mt. Prospect, Ill. Alkylated derivatives of aminocoumarin and hydroxycoumarin were a gift from Dr. P. Fromhertz, Max Planck Institut für Biophysik. Solvents in the surface pH studies were Spectro Grade (Fisher Scientific Co., N.J.).

Analytical Silica Gel 60 thin layer plates were obtained from EM reagents; Silica Gel G plates were from Supelco.

Cultures

Axenic cultures of *Ochromonas danica* were grown in the chemically

defined medium of Aaronson and Baker (1959). Cells were cultured at a pH of 4.5 except where otherwise noted. The cells were grown at 23.5 ^{0}C in the dark with occaisional exposure to ambient light. They were normally harvested after 4 to 5 days growth.

When appropriate, the purity of cultures was monitored by plating them out on growth medium containing 2% agar, and on bacterial medium (Tryptose Blood Agar). The plates were then incubated at 23 and 30 ^{0}C for at least 10 days. Live cells were also monitored using phase contrast microscopy (Zeiss) or after staining with crystal violet using standard optics.

Harvest

O. danica cultures were harvested by centrifugation at 300 x g for 15 min at 4 °C. In some experiments, the cells were concentrated by passing them through the Millipore cassette system loaded with a 0.45 micron filter (Millipore Corporation) at a flow rate of 125 ml/min Whole cells were recovered from the retentate, and were washed by passing additional fresh media (1/20 - 1/30 of the original culture volume) through the system.

Flagellar Detachment and Isolation

In either procedure, cells were resuspended in fresh media to 1/20-1/30th of their original volume, and chilled for at least one hour on ice.

Flagella were detached by vortexing the chilled cells in capped

centrifuge tubes (approximately 40% filled) at top speed in a Vortex-Genie mixer (Scientific Industries, Inc., Springfield Mass) for eight three second bursts. In detaching flagella by this method, it is important that the sample strikes against the top of the cap during the entire procedure.

The vortexed cells were then centrifuged at 300 x g (at 4° C; 15 min.) to pellet intact and deflagellated cells. The resulting supernatant was centrifuged at 300 x g repeatedly until no evidence of pelleted cells could be seen (usually, 2x). The supernatant was then pelleted at 13,300 x g for 20 min. to yield pelleted whole flagella. Unless otherwise indicated, the flagella were resuspended and pelleted once or twice more to ensure a high degree of purity. In a typical experimental run, 10 liters of cultures yielded nearly 100 mg. of purified flagella (dry weight).

Glutaraldehyde Fixation of Flagella

In those experiments where flagella were fixed with glutaraldehyde, pelleted flagella were gently suspended in a 2% solution of glutaraldehye (w/v) in 0.225M cacodylate buffer, pH 6, at 4 °C. After one to two hours, they were pelleted and washed several times with fresh buffer.

Flagellar Membrane Isolation

Flagellar membrane was isolated by the method of Stern (1978). The flagellar pellet was resuspended in 50 - 100 volumes of TEM buffer (10 mM Tris, 1 mM EDTA, 1 mM mercaptoethanol, pH 7.5). Exposure of the whole flagella to pH 7.5 removes the membrane from the flagellar microtubule

network (axoneme); exposure to 1mM EDTA dissolves the axoneme (Stern 1978). The suspension was stirred for 15 minutes, then centrifuged at 17,300 x g for 20 minutes (2x). The supernatant was then centrifuged at 22,000 x g for one hour to remove the bulk of the mastigonemes (glycoprotein hairs found on the external surface of flagella). The resulting supernatant was then centrifuged at 113,700 x g for 75 min. (Beckman Ultracentrifuge Model L2-65B, 60 Ti Rotor). The pellet, membrane vesicles contaminated with mastigonemes, was resuspended in TEM and the mastigonemes were collected by centrifugation at 28,400 x g for 45 minutes. The supernatant was then pelleted again at 113,700 x g for 75 minutes to produce purified flagellar membrane.

Extraction of Lipids

Reagent grade solvents were freshly glass-distilled and all procedures were conducted under nitrogen unless otherwise indicated. Lipid weights were determined using the Cahn 26 Electrobalance when necessary. The three procedures used for extraction of lipids from flagellar and whole cell pellets are described below.

Chloroform:methanol (2:1) Procedure. Freshly pelleted whole flagella from *O. danica* were suspended three times in twenty volumes of solvent. If after extraction the pellet became powdery, a small amount of water was added to congeal the pellet. After removal of residue, the combined extracts were evaporated under reduced pressure to near dryness, dissolved in a small volume of extracting solvent, and stored under nitrogen.

Modified boiling isopropanol procedure (Kates 1972). The flagellar pellet was suspended in 1 ml of 1M NaCl to which was added 5 ml of boiling isopropanol. The mixture was held in an 80 °C water bath for 4 minutes, then pelleted for five minutes at low speed on a desktop centrifuge (International Clinical Centrifuge Model Cl, International Equipment Co., Needham, Mass.). The residue was re-extracted several times with 5 ml chloroform: methanol 1:1 (v/v). The combined extracts were then rotary evaporated to near dryness, and re-dissolved in 5 ml chloroform:methanol 1:1 (v:v). To the supernatant was added 4.5 ml of 0.2N HCL. After centrifugation, the upper methanol-water phase was removed and the lower chloroform phase was made slightly alkaline by the dropwise addition of 0.2N methanolic ammonium hydroxide. The sample was then evaporated to near dryness under nitrogen.

Modified Bligh and Dyer (1959) *Procedure*. The flagella pellet was resuspended to a known volume with water. Chloroform and methanol were then added in the correct proportions to form the one phase system containing chloroform:methanol: water, 5:10:4 (v/v/v) ("one phase Bligh-Dyer solvent"). Any undissolved residue after extraction was pelleted down and re-extracted twice (once for the experiments with ^{14}C-oleic acid). The solvent extracts were combined. Chloroform (5 ml for each 19 ml of one phase Bligh-Dyer solvent) and water (same volume as chloroform) were then added to break phase. Both upper and lower phases were separated and washed with fresh lower and upper phase solvent (respectively). The lower phase washings and the original extracts for the lower phase were

combined, concentrated, and stored under nitrogen. The upper phase contains most of the sulfolipid, and was discarded unless otherwise indicated.

Thin Layer Chromatography

Analytical Silica Gel 60 thin layer plates or Silica Gel G were used. The latter plates were used mainly in the labelled oleic acid experiments, since they permitted the easy removal of lipid bands. Plates were pre-washed in chloroform: methanol 1:1(v/v), dried, and then activated by heating to 110 °C for one hour before use. Mobile phase solvent mixtures were equilibrated for 45 minutes before the start of chromatography. Nonpolar lipids were separated in one dimension using ether:hexane:acetic acid (15:85:1, v/v/v) as the mobile phase. Occasionally, these proportions were altered to 30:70:1 (E:H:A). Polar lipids were separated via chromatography in the two dimensional system of Rouser et al. (1967): System 1, chloroform: methanol: aq. ammonium hydroxide (28%), 65:35:10; System 2, chloroform: methanol: acetone: acetic acid: water, 50:15:20:10:5. TLC was not conducted under nitrogen.

Isolation of Lipids by Preparative TLC

Lipids were visualized using either iodine vapor or autoradiography. If iodine was used, most of the sample was covered with a clean glass plate and only a small amount exposed to the vapor. The protected portion of the silica containing the lipids was scraped off and extracted, or transferred to

scintillation vials for counting.

Nonpolar lipids were extracted from silica with either chloroform: methanol 2:1 or chloroform:methanol:ether 1:1:1. Polar lipids were extracted with the original one phase Bligh-Dyer solvent (chloroform:methanol: water, 5:10:4). Isolations were performed under nitrogen.

Gas Liquid Chromatography

Gas liquid chromatography (GLC) was conducted on a Perkin Elmer 881 Gas Chromatograph using a flame ionization detector. The methyl ester derivatives of the fatty acids were prepared by either transmethylation with 2,2-dimethoxy-propane in dry methanolic HCL (3N) (Mason and Walker, 1964), by microesterification using boron trifluoride in methanol (as in Chen et al. 1976), or by dropwise addition of freshly generated diazomethane. The unusual acidity of the membrane lipid extract made acidification prior to use of diazomethane unnecessary.

For preparation of diazomethane, diazald (5.4g) in ether (50 ml) was added to a 100 ml round bottom flask in a 70° C water bath. A separatory funnel connected through a Claisen adpator was used for the dropwise addition of a solution containing ethanol (12.5 ml), water (6 ml), and KOH (2.5 g). The diazomethane so generated was distilled over with the ether and trapped in an ice-chilled recieving flask. An additional ether filled flask (in ice) was connected in series to trap the excess diazomethane. The procedure was conducted in specially designed glassware ("clear seal" joints) for diazomethane generation (Wheaton) in an efficient fume hood.

Fatty acid methyl ester derivatives were injected into an 8 foot column

of SP2330 (10% w/v) Chromosorb WAW (100-200 mesh). Column temperature was held at 180 °C for 2 minutes, then raised to 215 °C at a rate of 4°/min. Unknowns were identified by comparison of retention time with known chromatographic standards and/or coinjection of standards with samples.

<u>Adsorption of Free Fatty Acids Using Albumin</u>

Purified flagella were suspended in fresh medium (containing 0.02% sodium azide) and separated into two portions by volume. Samples were pelleted and the supernatants discarded. Pellets were resuspended (2x) in medium containing human serum albumin (HSA; 4 mg/ml) to a volume of 5 or 2.5 ml (corresponding to 0.018 or 0.046 µmol HSA per mg flagellar protein, respectively). Control samples were resuspended with 4-5 ml media alone. Samples stood at room temperature (with occasional swirling) for 45 minutes. Additional media was added to each sample prior to centrifugation at 13,300 x g (20 min) to permit better separation and recovery of the supernatant from the pellet.

The supernatants were transferred to solvent-washed glass flasks so that extraction could be conducted without transfer of material. The flagellar pellets were suspended in media/azide (usually, to a volume of 10 ml) and aliquots were removed for further analysis.

Lipids were extracted by the procedure of Bligh and Dyer (see "Extraction of Lipids"). Pentadecanoic acid was added as an internal standard to the supernatant and resuspended pellet of each sample prior to extraction. Lower phase lipids were concentrated under reduced pressure.

Time Study on Free Fatty Acids after Flagellar Detachment

O. danica was cultured and harvested as previously described. An ethanol solution of [10-^{14}C]-oleic acid was added to the cultures 18 hours prior to harvest ("18 hour growth"); 3 days prior to harvest ("3 days growth"); or immediately after harvest but prior to flagella detachment ("<2 hrs exposure"). The volume of added ethanol ranged between 100 - 200 µl in a total culture volume of 450 - 550 ml for the "18 hour growth" experiments. For the other experiments ("3 days growth" and "<2 hrs exposure"), 2 ml of ethanol was used. The added lipid was judged pure by autoradiography of a thin layer chromatogram in the one dimensional solvent system used for nonpolar lipids. This procedure confirmed the absence of substances other than free fatty acids, but would not distinguish between different individual fatty acids (*e.g.*, stearic *vs* oleic acid).

After harvesting, cells were resuspended to a volume of 25 ml (approximately 1/20 of the original volume) and chilled on ice for at least one hour. After gentle swirling, the sample was divided into two (unequal) portions by volume. The smaller control portion was kept on ice for the duration of the experiment, and sampled at timed intervals after gentle swirling. The experimental portion was vortexed for 8 three second bursts at top speed in a capped centrifuge tube on a Vortex Genie Mixer (see "Flagellar Detachment and Isolation").

At timed intervals, after the vortexing, aliquots of each sample were transferred into glass screw cap test tubes containing a pre-measured volume of chloroform:methanol, 1.25:2.5 (v/v, under nitrogen) for each

volume of aqueous sample. This extracts the lipids in a one-phase Bligh-Dyer solvent.

In the "18 hour growth" experiments, portions of each vortexed aliquot (and control, unvortexed cells) were also transferred to 1.8 ml capped microfuge tubes at timed intervals and pelleted for 30 seconds in a Beckman Microfuge B (at approximately 7000-9000 x g). The resulting aqueous supernatant (enriched in ECV and flagella for the vortexed sample, and ECV alone for the control) was separated from the pellet and transferred to the lipid-extracting solvent. In one of the two studies ("18 hours growth #1"), the remaining pellets were then resuspended with 500 µl of fresh media and transferred to the extracting solvent. Aliquots of (1) supernatant and (2) resuspended pellet (10 or 20 µl. of a total sample volume of approx. 2-3 ml) were transferred to scintillation vials for counting before the addition of sample to the solvent for extraction. In another experiment ("18 hours growth #2"), aliquots of the supernatant *before* extraction were counted. Only 1 ml of the supernatant was used for extraction to prevent the accidental withdrawal of pelleted material, and the pellets were not resuspended or counted

In the "3 day growth" experiment, nearly half of the vortexed sample was used to isolate flagella and cell bodies. Cell bodies were first pelleted for 15 min. at 300 x g on a Sorvall RC2B centrifuge. The pelleted cell bodies were resuspended with media to a volume of 7 ml; a portion was counted directly and an aliquot was extracted and studied. The supernatant was centrifuged for 20 min. at 13,300 x g to pellet the flagella. The supernatant from this step contains extracellular vesicles (ECV) and media.

The chlorform:methanol:water extracts were converted to two phases

by the addition of chloroform and water as previously described (modified Bligh-Dyer procedure). In one experiment, after breaking phase, aliquots of the upper methanol-water phase were pipetted into scintillation vials for counting. In all experiments, the combined lower phase lipids were evaporated under nitrogen and dissolved in a volume of 100 to 200 µl. of solvent (chloroform: methanol 1:1). One tenth of the sample volume was used for counting the total activity of the sample. A volume of the remaining sample was then spotted onto a pre-washed TLC plate (silica gel G) and the chromatogram developed in the one-dimensional solvent system for nonpolar lipids (ether:hexane:acetic acid, 15:85:1).

Autoradiography

The chromatogram was dried in a fume hood. No-screen X-ray film (Kodak XAR) was placed between the chromatogram and a clean glass plate, weighted down, and exposed for one to three weeks in the dark depending on the activity of the sample. The autoradiograms were then developed using Kodak GBX developer and fixer.

Isolation of Radioactive Lipids

Labelled lipids were located on TLC plates using the autoradiograms. The silica was then scraped into scintillation vials (for counting) or into solvent washed glass test tubes for extraction and further studies. Percent free fatty acids in the total lower phase lipids was calculated either by dividing dpms of the free fatty acid band by the dpms of the "total lower

phase lipids" (counted directly from an aliquot of the sample before TLC ["18 hour growth" experiments]), or by summing up the counts from all lipid bands visualized using autoradiography and using this fiqure as the "total lower phase lipids" (all other experiments).

Acid Hydrolysis of Lipids

In order to examine the incorporation of labelled fatty acid into ester lipids, samples were hydrolysed and the hydrolysate examined by TLC. The entire procedure was conducted under nitrogen. The sample was transferred to a small glass test tube and evaporated to dryness. One to two ml of a 2N solution of HCL was added to the test tube, and a marble was placed on top. The test tube was then placed in a boiling water bath for one hour. Chloroform and methanol were added to the test tube to form the one phase solvent system (Bligh-Dyer) and the lower phase lipids were recovered.

Liquid Scintillation Counting

Samples were counted on either the Beckman 150 LSB or the Beckman 9000 LSB Liquid Scintillation Counters. Samples were placed into plastic mini-scintillation vials containing three ml of ScintiVerse II Universal Cocktail (Fischer Scientific) mixture. In a few instances where the samples might have to be stored before counting, they were placed in larger glass vials with 15 ml of scintillation fluid for counting. Counting was usually performed for a length of time sufficient to result in a 2% error or less. Quenching was corrected using the external ratios method.

Surface pH Studies of the Flagellar Membrane.

The surface pH measurements and model membrane preparation were performed by Michael Heller unless otherwise indicated. Amino coumarin (1 mg.) was dissolved in 4 ml of chloroform: methanol 1:3 (v:v); hydroxycoumarin (6 mg.) was dissolved in 6 ml methanol.

Measurements were made on a Perkin-Elmer Fluorescence Spectrophotometer (MPF-2A) with excitation set at 366 nm. Filter #43 was used. Slit settings were varied to reduce background and ranged from 6-10 for both excitation and emission slits. Emission scanning was between 400-600 nm. The emission peak for both indicators was at 450 nm.

To determine the pK of the hydroxycoumarin indicator at the membrane surface, a 3 ml dispersion of the membrane preparation was adjusted to pH 11.7 with 0.1 N NaOH. After scanning the preparation to determine a minimal blank, 5 µl of the indicator solution was added to the sample and scanned. The pH was then adjusted to 11.9. The indicator at this pH was completely dissociated since the peak height and emission spectrum was unchanged from its previous levels. The preparation was then titrated by the addition of increments of HCl to pH 1. The fluorescence peak height was determined periodically during the titration, which was considered complete after it ceased to change in the presence of added acid.

The peak heights were measured, and the percent dissociation calculated after corrections for volume change. Percent dissociation was found by dividing each peak height by the peak height at the plateau (total dissociation of the indicator). These calculated values were plotted and the pH corresponding to 50% dissociation was considered to be the pK.

A similar procedure was used for the hydroxycoumarin indicator. A 3 ml dispersion of vesicles (pH 4.1) was adjusted to pH 6.6, where the indicator was completely dissociated. This was used as the blank. Hydroxycoumarin indicator solution (20 µl) was then added, and the solution was scanned at various pH's to pH 0.8. The points were calculated, plotted, and the pK was determined as before.

Protein Assay

Protein was measured by the method of Lowry et al. (1951), using lipid-extracted bovine serum albumin as a standard.

Azure A Assay for Sulfolipid

The procedure of Kean (1968) was used. Samples (<0.1 ml) were pipetted into glass screw cap test tubes. To each tube was added 5.0 ml each of chloroform:methanol 1:1 (v/v), 0.05 N sulfuric acid, and 1 ml of Azure A solution. The tubes were capped, vortexed for 30 seconds each, and centrifuged for 5 minutes to separate the two phases. The upper phase was quickly removed by means of a pasteur pipette connected to a water aspirator. The absorbance of the lower phase at 645 nm. was read on a Gilford spectrophotometer. Concentrations were determined from a standard curve using sodium dodecyl sulfate (SDS). Concentrations of *O. danica* sulfolipid assayed in this way were then divided in half to obtain molar quanities, due to the presence of two sulfate groups on the molecule.

RESULTS

Lipase Inhibition

Quantitation of the Free Fatty Acid Fraction. It had been shown that free fatty acids (FFAs) constitute 12.3 mol% of *O. danica's* flagellar membrane lipids (Chen et al. 1976). These results are confirmed in the present work, which attempts to assess whether or not the FFA's resulted from artifacts related to flagellar scission, lipid extraction, or both. The following results relate attempts to inhibit or demonstrate the absence of a putative lipase. A study on flagellar scission as a potential source of free fatty acids will be dealt with separately.

Although extraction using polar solvents such as methanol is sufficient to denature many lipolytic enzymes, there are cases (particularly in plant tissue) in which these enzymes are unusually stable in the presence of denaturing solvents. Solvent-activation of lipid degradation has been well described (Brockerhoff and Jensen 1974). In order to establish the presence or absence of free fatty acids in the flagellar membrane *in vivo*, it was deemed necessary to establish whether or not the fatty acids isolated from the membrane preparation were produced by lipase activity during extraction of the lipids.

A preliminary extraction step using boiling isopropanol is known to denature many lipases otherwise activated during extraction (Kates and Eberhardt 1957; Kates 1972). Therefore, the quantity and composition of the

free fatty acid fraction after this treatment was compared to those of untreated samples. Extraction using boiling isopropanol failed to eliminate the free fatty acid fraction from whole flagella (Table 1), nor did it significantly depress this fraction: for equal quantities of whole flagella, 0.34 mg FFA, treated *vs* 0.32 mg FFA, untreated were found. The free fatty acids represented 0.7% of the weight of the total of each of these samples. Calculations using the data from Chen et al. (1976) predict FFAs to account for 1.9% of the total flagellar weight. Procedural differences may in part explain these results.

One difference between the boiling isopropanol/high salt treatment and the "control" extraction was the use of an acidified Bligh-Dyer procedure in the former. In this case, the free fatty acids were separated from other components by their partitioning into the lower chloroform/methanol phase. The discarded upper phase would contain most of the chlorosulfolipids, which comprise about 71% of the membrane lipids on a molar basis. Thus, the extracted "total lipid weights" from the treated sample (2.8 mg in the absence of most chlorosulfolipids, or approximately 10 mg when corrected to reflect the discarded lipids) and the 2:1 chloroform: methanol extraction (11.0 mg, containing sulfolipids) are comparable.

Glutaraldehyde is a crosslinking reagent routinely used in fixation of samples for electron microscopy. This agent is not expected to directly inactivate a putative lipase, since many glutaraldehyde treated enzymes and proteins have been shown to retain their activities. However, crosslinking of the flagella before extraction would minimize contact between a putative lipase and substrate by preventing the extraction of the lipase into the organic solvent (the proteins are attached to the flagella rather than

TABLE 1

Comparison of Extraction Procedures Designed to Inhibit Putative Lipase Activity: Quantities of Isolated Free Fatty Acids.

	Treatment and Extraction	Flagella	Total Lipids	FFA	FFA : Flagella	FFA : Total Lipids
		weight (mg)			weight %	
A.	2:1 Chloroform: Methanol (1 phase)	48.2	11.0	0.32	0.70	3.1
B.	Boiling Solvent, High Salt; Bligh and Dyer (2 phase)	48.2	[10.0]	0.34	0.71	[3.4]
C.	Glutaraldehyde Fixation; 2:1 Chloroform:Methanol	59.8	1.3	0.13	0.22	9.9

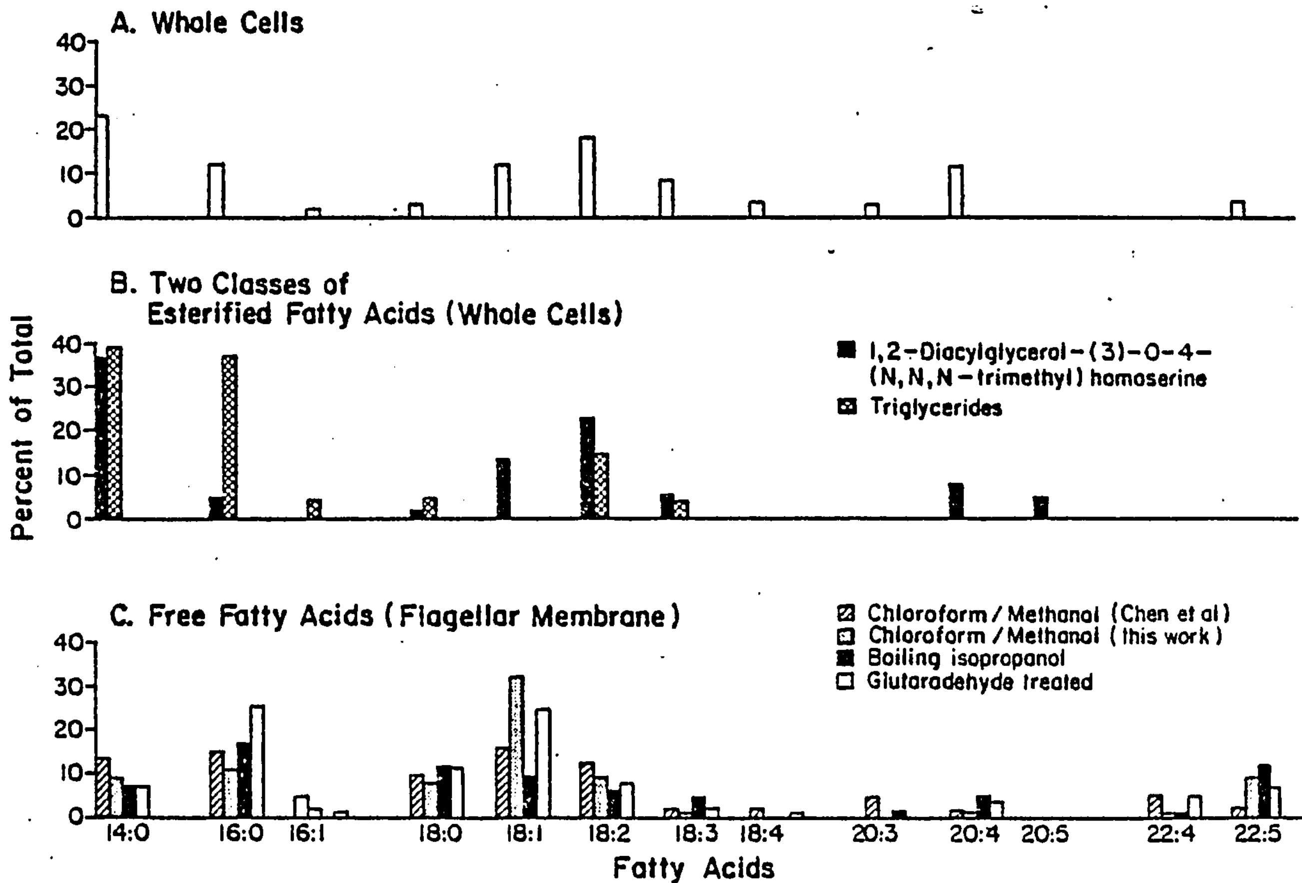
A. Flagella were pelleted and extracted two times at 4 °C with twenty volumes of chloroform: methanol 2:1 (v/v). This single-phase solvent system extracts the sulfolipids which represent 71% of the membrane lipids on a molar basis (Chen et al. 1976). Free fatty acids (FFAs) were isolated by thin layer chromatography; the FFA band was scraped and eluted. Weights of total lipid extracts and of FFAs were obtained using a Cahn 26 electrobalance. **B.** The pellet was suspended in 1 ml of 1M NaCl; extracted for four minutes with 5 ml of boiling isopropanol in an 80 °C water bath; then twice extracted with chloroform:methanol 1:1. The extract was subsequently broken into two phases, and the lower phase was worked up as described above. The upper phase was discarded, causing the selective loss of the sulfolipids. The data for total lipids [10 mg] and for wt. % of FFAs in flagella [3.4%] has been estimated from the actual weight of the lipid extract (2.8 mg) in the absence of sulfolipids. **C.** The pellet was suspended in 10 ml deionized water to which was immediately added a 2% solution of glutaraldehyde in 0.225 M cacodylate buffer (pH 6.0). The sample was held at 4 °C for 1 hour before repelleting. It was then extracted with chloroform:methanol 2:1 as described in **A** above.

extracted).

Treatment of the flagella with glutaraldehyde prior to chloroform: methanol 2:1 (v/v) extraction resulted in a decrease in the total fraction of extracted lipids. The pellet itself became brittle. The levels of FFA to flagella weight (0.22) were about one third of those of controls (0.77). However, FFAs were a greater percentage of the total extracted lipid weight in the glutaraldehyde treated (9.9%) as compared to the control sample (3%), presumably because of the inefficiency of sulfolipid extraction. Korn and Weisman (1966) also reported a higher loss of nonpolar (as compared to polar) lipids during alcohol extraction (and subsequent dehydration) of glutaraldehyde-fixed *Acanthamoeba* sp. cells. In *Acanthamoeba*, the loss of nonpolar lipid counts was quantitative; however, 16% of the polar lipids remained in the crosslinked pellet after this treatment. The chlorosulfolipids of *O. danica* are considerably more polar than most phospholipids, and are thus expected to be much less well extracted.

Composition of extracted free fatty acids. It is typical of membrane lipids that the composition of the chains in a given lipid class is a characteristic of the membrane that contains the lipid. A free fatty acid fraction of a given membrane might be expected to have a unique composition. This composition could be different from that of ester lipids found in the same cell; indeed each class of ester lipids should have its own unique composition. The composition of free fatty acids extracted by each of the three procedures described above was compared with the fatty acid compositions of fractions of ester lipids from *O. danica* (Figure3). Saponified whole cell lipid extracts (Gellerman and Schlenck 1965), tri-

FIGURE 3



Comparison of the flagellar free fatty acid (FFA) composition with fatty acids from other fractions. **A.** Total saponified fatty acids of whole cells (Gellerman and Schlenck 1965). **B.** Two classes of esterified lipids from whole cells: THAGE (Brown and Elovson 1974), and the triglycerides (Roethel and Haines 1978). **C.** Flagellar membrane FFA in the presence or absence of treatments to inhibit a putative solvent-activated lipse (Chen et al. 1976, and this work). The extraction procedures for **C** are described in Table 1. Other procedures are given in "Experimental Procedures".

glycerides (Roethel and Haines 1978), and THAGE [1,2-diacylglycerol-(3)-O-4-(N',N',N'-trimethyl)-homoserine, Brown and Elovson (1974)] were compared.

The flagellar FFA fraction varies in its composition from those esterified lipids whose composition has been determined. For example, the dominant flagellar free fatty acids are oleate (15-25%) and palmitate (15%). In contrast, the major fatty acid in the total lipids and THAGE is myristate (20-40%), and palmitate (40%) is also a major acid in triglycerides; oleate (10-15%), linoleate (20%), and arachidonate (10%) are present in moderate proportions (Gellerman and Schlenck 1965; Roethel and Haines 1978; Brown and Elovson 1974). The pattern of long chain polyunsaturated fatty acids for each class of lipids just mentioned is also unique and reproducibly characteristic. Thus, the free fatty acids have a composition that differs from that of other lipid components of the whole cell.

Albumin adsorbtion of flagellar free fatty acids

Albumin adsorption was used to determine whether or not free fatty acids could be demonstrated in flagellar preparations prior to solvent extraction. The distribution of FFAs between pellet (containing flagella) and supernatant (with or without albumin) is shown in Table 2. The quantity of total FFAs does not seem to vary significantly between albumin-exposed (0.21, 0.16 µmol FFA/mg flagella protein) and non-exposed (0.18 µmol FFA/mg flagella protein) samples. However, distribution of the fatty acids between pellet and supernatant phase was affected by albumin treatment.

In non-exposed samples, 4-5% of the total FFAs were found in the

Table 2

Albumin Adsorption of Free Fatty Acids from Flagella

Flagellar Protein (mg/ml)	Added Albumin (µmol /mg flagellar protein)	Total FFA (µmol /mg flagellar protein)	Flagellar Free Fatty Acids: Pellet (P) (µmol)	Supernatant (S) (µmol)	Supernatant Albumin alone * (µmol)	Total (S+P) (µmol)	% FFA in S
A. 14	0.000	0.18	3400	160	0.00	3500	4
B. 10	0.018	0.21	1800	380	0.25	2200	17
C. 5	0.046	0.16	370	180	0.15	560	33

Aqueous solutions were preserved with 0.02% (w/v) sodium azide. **A.** Whole flagella were suspended (2x) with 5 ml culture media. **B**, **C**. Flagella were suspended (2x) in a solution of defatted human serum albumin (4 mg HSA/ml media); suspension volume was 4 ml for **B**, 2.5 ml for **C**. Samples were pelleted (13,300 x g, 20 min) after standing at room temperature with occasional swirling for 45 minutes. Additional media (4-5 volumes) was added prior to centrifugation to aid in separation of pellet and supernatant. The combined supernatants were extracted by the method of Bligh and Dyer (1959). The pellets were resuspended in media and extracted. Internal standard (pentadecanoic acid) was added to all samples during extraction. Lipids were concentrated, methylated using diazomethane, and quantitated using gas chromatography.

* Residual FFAs found in extracts of the HSA supernatant (flagella absent)

supernatant. It had previously been noted that a significant portion of isolated *O. danica* extracellular vesicles (sulfolipid and protein) dissolves with repeated pelleting and resuspension, particularly at alkaline (20%) and neutral (10%) pH (El Maraghy 1982). After albumin treatment, 17 - 33% of the FFAs were found in the supernatant (Table 2); this represents a net transfer of FFAs to the supernatant phase. The quantities of residual FFAs that could be extracted from "delipidated" albumin in the absence of flagellar membrane were low (< 0.1% of the amounts found in the flagella fractions). Sulfolipid was also transferred into the supernatant in the presence of albumin, as shown by thin layer chromatography and Kean assay. Sulfolipid was undectable (by Kean assay) in the quantity of albumin used in these experiments

The fatty acid composition of the supernatant (in the presence or absence of albumin) was similar to the composition of the pellet for each sample of whole flagella. Morphology of the albumin exposed and control flagellar membrane was judged unchanged by treatment based on transmission electron microscopy.

In conclusion, the net transfer of flagellar free fatty acids from the pellet to supernatant phase after exposure to albumin demonstrates that the FFAs are not produced by a solvent-activated lipase.

Flagellar Detachment Study

Although lipase activity had not previously been associated with deflagellation (or "de-ciliation") of flagellated (or ciliated) cells, an investigation of time-dependent appearance of fatty acids after flagellar

scission was conducted. Cells were cultured in the presence of [10-^{14}C]-oleic acid for 18 hours ("18 hour growth") or for 3 days ("3 day growth"). Label was also added to cells that had been cultured, harvested and resuspended in its absence, 15 minutes prior to the flagellar detachment procedure ("<2 hour exposure").

Distribution of Labelled Material Among Cell Fractions After Initial Harvest. The unharvested cultures are a mixture of whole cells, extracellular vesicles (ECVs), and culture media. After 18 hours growth on [10-^{14}C]-oleic acid, approximately 92% of the introduced counts remained in the unharvested cultures. Harvesting concentrates the whole cells and removes most of the ECVs accumulated by the cells during their 4-5 day growth, which remain in the discarded supernatant. Nearly 30% of the total counts (after 18 hours exposure to label) for the unharvested culures were associated with the supernatant after pelleting the cells (Table 3). The ECVs have a lipid composition similar to that of the flagellar membrane (El Maraghy 1982), and it seems likely that the supernatant counts are associated with (adsorbed to) these vesicles. As would be expected, the relative amount of label associated with the supernatant and pellet is dependent on the length of time the label was added prior to harvest, and/or to the stage of growth of the cultures. Thus, a higher percentage of label is found associated with the pelleted whole cells after 3 days growth with label (87%) than after 18 hours of growth (72%).

Distribution of Labelled Material Among Cell Fractions After (±) Flagellar Detachment Treatment. Table 4 shows the distribution of

TABLE 3

DISTRIBUTION OF COUNTS AMONG CELL FRACTIONS AFTER GROWTH ON LABELLED OLEIC ACID:

Initial Cell Harvest

	Time of growth after addition of labelled oleic acid	
	18 hours	3 days
	Dpm/ml; (Percent of total dpm in fraction)	
Pellet	9,021.2 (72%)	3,844.0 (87%)
Supernatant	3,575.5 (28%)	574.4 (13%)
Total	12,596.7 (100%)	4,418.4 (100%)

Cells were harvested after 4 1/2 days growth by centrifugation at 300 x g for 15 min. A sample of the cultures before centrifugation (total), and the supernatant after centrifugation were counted. The pellet counts were calculated from the difference. Growth medium was used as a blank; counts due to blank have been subracted from the tabled data.

TABLE 4

DISTRIBUTION OF COUNTS AMONG CELL FRACTIONS AFTER GROWTH ON LABELLED OLEIC ACID:

With and Without (±) Deflagellation

	A. < 2 hrs	B. 18 hours		C. 18 hours		D. 3 Days
	Deflagellation					
CELL FRACTION	+	-	+	-	+	+
	Total dpm					
Whole cells	234,000 (82%)	127,000 (97%)	120,000 (93%)	-	-	728,000 (98%)
ECVs (± Flagella)	- (18%)	3,700 (3%)	8,800 (7%)	7,100 (2.9%)	10,500 (4.2%)	- (2%)
Flagella	>27,200 (10%)	-	-	-	-	>9,000 (1%)
ECVs	23,000 (8%)	-	-	-	-	9,600 (1%)
100%	285,000	130,000	126,000	246,000	245,000	746,600

Samples were inoculated with [10-^{14}C]-oleic acid for 18 hrs (**B, C**) or 3 days (**D**) prior to harvest. **A** was grown concurrently with **D**, but in the absence of label (which was subsequently added 15 minutes prior to deflagellation). After harvest, the pelleted whole cells were resuspended in cold media, chilled on ice for at least 1 hr, then divided into portions to serve as deflagellated (+) and control (-) samples. In **B**, samples were pelleted for 30 sec in a Beckman Microfuge B after treatment, thus separating the cells from the supernatant (containing extracellular vesicles, ECVs ± flagella). Aliquots of the supernatant and the resuspended pellet were counted.

TABLE 4

(Continued)

The procedure was essentially the same in **C** except that pellets were discarded. In **A** and **D**, only "deflagellated" samples were fractionated. Cells and (crude) flagella were harvested as described in "Experimental Procedures". They were resuspended in media and aliquots counted. The supernatant remaining after pelleting of the flagella (enriched in ECVs) was also counted. Thus, the supernatant after pelleting of whole cells was not separately counted as for **B** and **C**. The percent of the total counts represented by the sum of flagella + ECV counts (17.8%, **A**; 2.5%, **D**) is included in the table for purposes of comparison. Actual counts for the flagella may be somewhat higher than noted here. The flagella were suspended in 1 ml media and an aliquot was counted, however the volume occupied by the pellet was ignored when extrapolating the counts from the aliquot.

counts between various cell fractions was also tested after the treatment (±) for flagella detachment. The whole cells pelleted in Table 3 were first resuspended to a volume of 20-30 ml, chilled for at least one hour, and (±) vortexed as described above prior to the centrifugation shown in Table 4. This once more separates the cells (whole or deflagellated) from their supernatant (containing ECV, media, and ± flagella).

In Table 4, the ratio of counts present in the supernatant to those in the pellet was lower than after the initial harvesting (Table 3) for each sample. This was true for both deflagellated and control samples. For example, in one control sample at this stage, only 3% of the total counts of the unseparated mixture (supernatant + pellet) were contributed by the supernatant. In contrast, during the initial cell harvest of this sample, over 20% of the total counts were due to the supernatant. This is consistant with the removal of most of the cultures' original ECVs during harvest. It is likely that the counts in the supernatant obtained after initial cell harvest are due to new ECV formed by the cells after harvest. However, it would be possible for some of these counts to represent residual ECVs trapped among the original pelleted cells, or contamination from small amounts of whole cells from the pellet.

The supernatant of cells (from the same sample) given the flagellar detachment treatment represented 7% of the total counts before centrifugation, or more than double the amount found in the control sample. This is consistent with the transfer of flagella from the pellet (attached to cells in control samples) to the supernatant after the vortexing treatment.

Labelling Patterns of Lower Phase Lipids. Oleic acid is taken

up by *O. danica* (Mooney et al. 1972), is a major component of flagellar membrane FFAs (Chen et al. 1976), and is not on the direct pathway for sulfolipid biosynthesis (Elovson 1974). The five major labelled spots (after <2 hrs and 3 days growth) after chromatography of *O. danica* lower phase lipids are shown in Figure 4. An autoradiogram of the supernatant (enriched in flagella and ECVs; 18 hours growth on label) is shown in Figure 5.

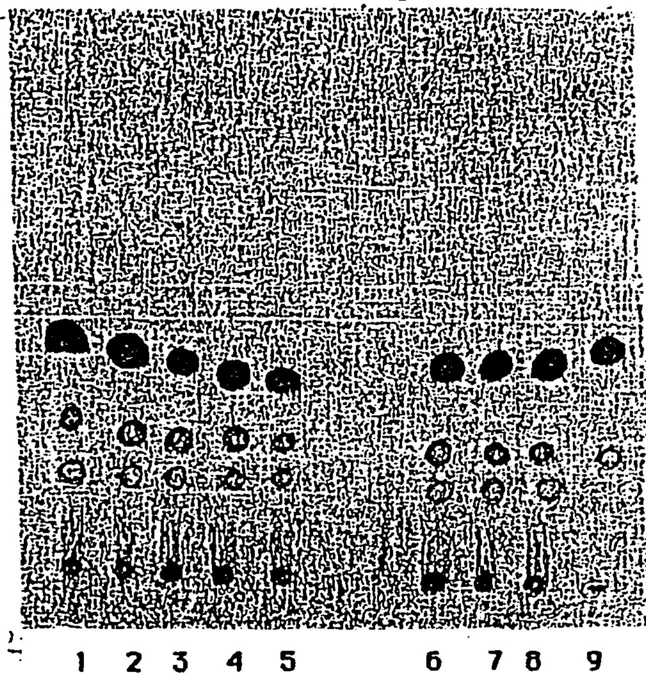
The time of cell growth after exposure to label had a significant effect on the percent label recovered as FFA (Tables 5, 6). In cells (whether or not pelleted after treatment) exposed to label 15 minutes prior to (±) deflagellation (Figure 4; Table 5), about 90% of the counts were recovered as free fatty acids, with 4-6% appearing at the origin, and trace amounts (<1%, ~1%, 2-3%) appearing as unknown*1, unknown*2, and sterol, respectively. Isolated flagella showed higher amounts of label appearing as free fatty acid (96%) and lesser amounts at the origin (<3%). The other lipids (unknown*1, unknown*2, and sterol) may were not detected under these conditions. Components representing 0.5% or less of the total would be indistinguishable from background counts.

After three days growth on label (Figure 4; Table 6), the major class of labelled lipid remained at the origin (70-80% of lower phase counts); FFAs represented only trace (1-3%) amounts. Unknown*1 now accounted for 20-30% of the total lower phase counts. Isolated (crude) flagella showed higher amounts of FFA counts after 3 days (12.8%) compared to the whole cells (1-3%). Equal amounts of material at the origin (71%), and much lower quantities of unknown*1 (<3%) were present. The low quantity of unknown*1 suggests that it could be a contaminant from the whole cells, especially since it also appeared to be absent from the flagella-

Figure 4

<u>Auroradiogram of Lipids from *O. danica* After <2 Hours and 3 days Growth in the Presence of Labelled Oleic Acid</u>

A. <2 hours growth



B. 3 days growth

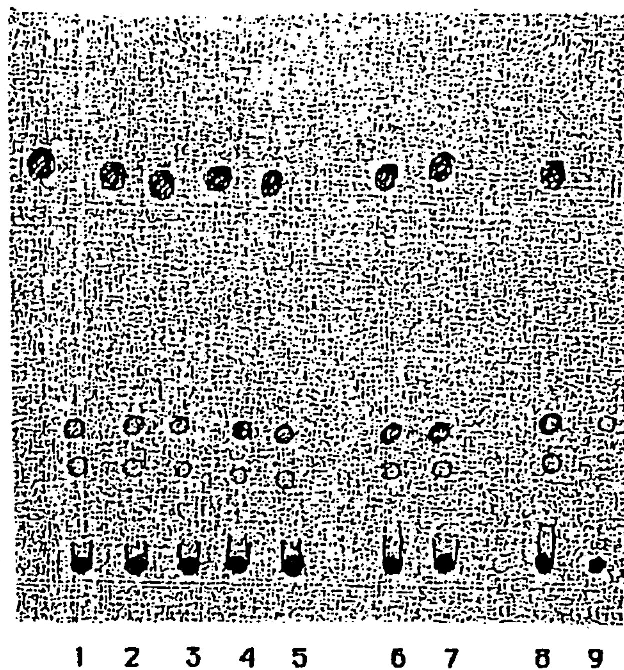


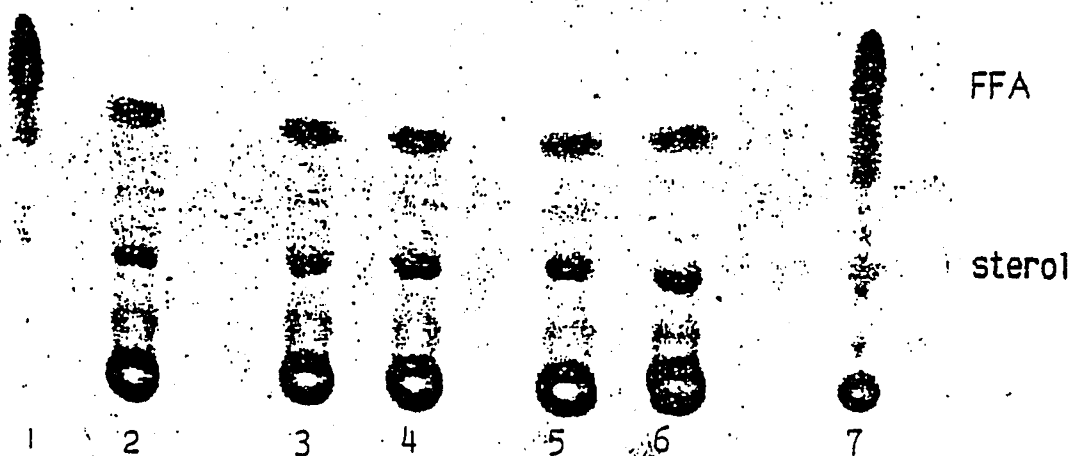
Figure 4

(Continued)

Labelled lipids from lower phase extracts of *O. danicia* separated by thin layer chromatography (mobile phase was ether:hexane:acetic acid 15:85:1). Labelled oleic acid (0.27 µCi, **A**; 0.6 µCi, **B**) was added to cultures (15 minutes prior to deflagellation, **A**; 3 days prior to harvest, **B**) which were subsequently harvested and deflagellated as described above. Lanes *1, 2:* aliquots of cell mixture (not deflagellated) at start of experiment. Lanes *3-7:* aliquots of deflagellated cell mixture, taken at 2, 4, 8, 20, 60, and 120 minutes after *start* of the deflagellation procedure. Lane *8:* pelleted cells from the cell mixture after deflagellation. Lane *9:* pelleted flagella from cell mixture after deflagellation.

Figure 5

Autoradiogram of Lipids from *O. danica* Supernatant After 18 Hours Growth in the Presence of [10-^{14}C]-Oleic Acid.



Autoradiogram of lipid extracts containing flagella + extracellular vesicles (ECV) from the supernatant of deflagellated cells (harvested 18 hours after addition of labelled oleic acid). Lipids were separated by thin layer chromatography using ether:hexane:acetic acid (15:85:1). The positions of FFA and sterol are shown. Samples were collected and pelleted over a 30 minute time period from the start of deflagellation. Control supernatants (not deflagellated; ECV only) showed essentially the same pattern of labelling. Pellets (whole or deflagellated cells) or uncentrifuged samples showed lower amounts of free fatty acids, and had substantial amounts of unknown #1. Identity of spots: 1, oleic acid; 2-6, lipid extracts of *O. danica* supernatant in deflagellated cells, obtained 6, 10, 14, 20, and 30 minutes (respectively) after start of de- flagellation treatment; 7, oleic acid co-chromatographed with a small portion of extract from 5.

TABLE 5

COMPOSITION OF MAJOR LABELLED LIPIDS APPEARING ON AUTORADIOGRAM, AS % TOTAL DPM.

"2 hours exposure" to labelled oleic acid

	DEFLAGELLATED SAMPLES			
	A. Cell Bodies + Flagella + ECV		B. Cell Bodies	C. Flagella
	Time after start of treatment		Time after start of treatment	
	1 min	60 min	>60 min	>60 min
	Dpms per labelled spot			
unknown 1	12.8 (0.6%)	14.7 (0.6%)	15.0 (0.7%)	- -
FFA	2,067.1 (89.8%)	2,432.0 (91.1%)	1,873.0 (89.9%)	1,292.4 (96.0%)
unknown 2	70.7 (3.1%)	44.1 (1.7%)	41.0 (2.0%)	17.4 (1.3%)
sterol	27.3 (2.7%)	17.1 (0.6%)	41.8 (2.0%)	- -
origin	124.6 (5.4%)	122.8 (4.5%)	111.6 (5.4%)	35.7 (2.7%)
Total	2302.4 (100%)	2670.7 (100%)	2082.7 (100%)	1345.5 (100%)

Table 5. Five day old cultures of *O. danica* were harvested, resuspended in media, and chilled in preparation for deflagellation as described in "Experimental Procedures". Labelled [10-^{14}C]-oleic acid (0.3 µCi in 200 µl ethanol) was added to the cells, and they were gently swirled. Flagella were detached from the cells about 15 minutes later. **A.** Aliquots of the mixture were removed into solvent 1 min and 60 minutes after the start of treatment. **B.** In addition, about half of the total sample was transferred into a capped centrifuge tube, and cells were pelleted (300 x g, 15 min). The pelleted material (cell bodies; about 0.1 µCi) was resuspended and an aliquot was extracted. **C.** The supernatant (enriched in flagella + ECV) was then spun at 13,300 x g for 20 min, yielding a pellet consisting mostly of flagella. The pellet was resuspended and extracted, and a portion was counted without extraction. This preparation of flagella is more crude in quality that normal, since it would usually have been resuspended and pelleted once or twice more. An aliquot of the supernatant remaining after pelleting the flagella (enriched in ECV) was counted directly. The samples were extracted and lipids separated using TLC as described in "Experimental Procedures". The labelled bands of silica were scraped directly into scintillation vials and counted. The "total" for % composition (by dpm) was found by summing the counts from individual labelled bands on the autoradiogram for each sample.

TABLE 6

COMPOSITION OF MAJOR LABELLED LIPIDS APPEARING ON AUTORADIOGRAM, AS % TOTAL DPM.

Three days growth in the presence of labelled oleic acid.

		Control samples		Deflagellated samples				
		A. Cells+ECV		B. Cells+ Flagella+ECV			C.Cells	D.Flagella
		0 min	0 min	3 min	8min	60min	>60mi	>60min
unknown 1	dpm	383.1	456.0	221.8	232.5	325.0	330.3	26.5
	%	20.3	30.2	13.6	14.7	20.5	17.8	3.7
FFA	dpm	27.4	27.0	20.0	45.5	42.4	57.0	92.3
	%	1.5	1.8	1.2	2.9	2.7	3.1	12.8
unknown 2	dpm	85.4	89.6	87.5	69.0	65.9	120.7	69.7
	%	4.5	5.9	5.4	4.4	4.1	6.5	9.7
sterol	dpm	18.1	13.9	17.9	14.3	14.7	24.4	7.7
	%	1.0	0.9	1.1	0.9	0.9	1.3	1.1
origin	dpm	1368.7	925.4	1282.5	1220.1	1112.9	1328.3	523.7
	%	72.6	61.2	78.7	77.2	72.6	71.4	72.7
Total	dpm	1882.69	1521.9	16291.6	1581.4	1260.9	1860.7	719.7
	%	100	100	100	100	100	100	100

Table 6. Five day old cultures of *O. danica*, grown for 3 days after the addition of 0.59 µCi of [10-^{14}C]-oleic acid in ethanol, were harvested, resuspended in cold media and chilled for 1 1/2 hours. Samples (**A, B, C, D**; described below) were pipetted into solvent; lipids were extracted, separated, and counted as described in "Experimental Procedures". Total counts were calculated from the sums of the individual lipid bands. **A.** Aliquots (2) of the resuspended cells. The remainder of sample was then (immediately) deflagellated. **B.** Aliquots of the deflagellated cell mixture were pipetted into solvent at timed intervals over a one hour period. Timing was from start of treatment. Cells were gently swirled before sampling. **C.** Deflagellated cells (about 50% of total sample) were pelleted and resuspended; aliqouts were counted and extracted. **D.** Flagella were obtained from the supernatant in **C** (centrifugation at13,300 x g, 20 min) then resuspended in fresh media and extracted.

enriched supernatant of another sample (18 hours growth; Figure 5), whereas it was present in whole or deflagellated cells after 18 hours growth (data not shown).

Are the low levels of labelled free fatty acids after 3 days growth due to extensive metabolism and *de novo* synthesis, or has the acyl chain been incorporated into ester lipids essentially unaltered? And what effect would this have on the ability for lipolysis to be demonstrated, should it exist?

If the compound running with the same R_f as cholesterol is indeed sterol, fatty acid oxidation of some label has taken place. A constant 1% labelling of sterols in both whole cells and flagella was seen after 3 days growth. Certain lipids, such as the pigments (typically running just below the solvent front in this system) appeared to be totally unlabelled. Two dimensional TLCs in a polar solvent system failed to show much, if any labelled glycerol remaining at the origin, or evidence for labelling of sugars, although this may have been due to their removal into the upper of the two phase extraction system. Labelled sulfolipd was present, futher indicating *de novo* synthesis of fatty acid (Elovson 1974).

Thus, the polar lipids (from whole cells) remaining at the origin after TLC in a non-polar solvent system probably represent a mixture of sulfolipid and other polar compounds. Acid hydrolysis followed by re-extraction and chromatography in the non-polar solvent system of total whole cell lipids (3 day sample) resulted in a change in the distribution of label. The free fatty acid fraction increased to 65% (from 3%) and the material at the origin decreased to 8% (from 70%). It should be noted that much if not all sulfolipid would be hydrolyzed to diol under these conditions. Diols partition into chloroform/methanol, but are fully resolvable from FFA in the

chromatographic system used. Appreciable amounts of unknown*1 were still present after acid hydrolysis in this sample.

Elution and re-chromatography of material from the origin of another sample (18 hours growth; whole cells) did not generate FFAs or other labelled products, but acid hydrolysis prior to chromatography caused the appearance of additional labelled FFAs on autoradiograms. Attempts to determine whether the material from unknown*1 also broke down to generate FFAs were unsuccessful, perhaps due to the small amounts of available material in the 18 hour sample. Autoradiograms of samples after elution and re-chromatography of either control or hydrolyzed sample were indistinguishable from solvent blanks. The time course of the appearance of this material suggests it is not synthesized directly from oleic acid.

The results of hydrolysis suggest that a pool of the total labelled lipids in whole cells are esterified, polar, and can potentially generate FFAs. Thus, this method is valid in the demonstration of whether or not deflagellation causes lipolysis.

Effect of Deflagellation Procedure on Free Fatty Acid Levels with Time. Flagella could not be isolated quickly enough to permit a meaningful time study on the effect of the deflagellation procedure on unesterified fatty acid levels. Therefore, studies were first conducted on "unseparated mixtures" (ie, whole cells+ECV *vs* deflagellated cells+flagella +ECV), and later, on the supernatant remaining after pelleting of whole cells (ECV *vs* flagella+ECV).

The data (Table 6 and additional data) fail to show a significant increase in free fatty acid levels for unseparated mixtures. The free fatty

acid levels after three days growth on labelled oleic acid were low in all cases (1-3%) for the cells, and the control levels (averaging 27 dpm above background, or 1.7% of the total lower phase lipids) fell within the levels of the deflagellated cells (20 - 45 dpm above background, or 1.3-2.9%). In another experiment (18 hours*1; not shown here), the levels of free fatty acid for control and deflagellated cells ranged from 4.5-5.5% of the total lower phase lipids over a one hour period.

The free fatty acid levels also remained constant over time in the supernatant from control and deflagellated cells (Table 7). Free fatty acids represented 4-5% of the total *unextracted* counts of the supernatant in cells which had been deflagellated, and levels here were actually slightly lower than for the untreated cells.

The unextracted total supernatant counts showed less variability than the total lower phase lipid counts, and were found to be a more accurate way to portray the free fatty acid levels. The lower phase lipid counts decreased over time for many of the control and deflagellated samples in the experiments. In the three day experiment (and in an 18 hour experiment not shown here), the decrease seemed to occur primarily from material at the origin. Thus, some of the material at the origin may be metabolized in both control and deflagellated cells during the time course of the experiment, at 4 °C. This may also be happening in flagella and ECV.

Loss of lower phase lipid counts could occur through partitioning of label into the upper phase during extraction, or by increased association with the insoluble residue. Complete extraction of all sulfolipid from the cell residue is extremely difficult, as has been noted previously (Haines 1965; Mooney 1973).

TABLE 7

Counts Appearing as Free Fatty Acid in the Supernatant of Cells With and Without (±) Deflagellation

18 hours growth after addition of labelled oleic acid

		CONTROL (ECV)			DEFLAGELLATED (Flagella + ECV)				
		Time after start of treatment			Time after start of treatment				
		6 min	14 min	30 min	6 min	10min	14 min	20 min	30 min
FFA	dpm	572.8	447.7	432.2	447.7	447.8	456.2	504.4	444.4
FFA / mean total supernatant counts	%	8.3	6.5	6.2	4.4	4.4	4.5	5.0	4.4
Total lower phase lipid	dpm	4,793.78	3,654.3	4,958.6	7,245.9	6,053.0	5,241.7	5,602.5	4,048.8
Total lipids/mean total supernatant counts	%	69.3	52.8	71.7	71.4	59.6	51.7	55.2	39.9
Total counts in supernatant (before extraction)	dpm	6,708.2	7,021.0	7,021.0	10,147.5	9,312.9	10,355.4	10,771.2	10,147.5

Table 7. Labelled oleic acid was added to 3 day old cultures, which were harvested 18 hours later. Cultures contained 2.3μCi immediately prior to harvest. Deflagellation and prior steps were as previously described. Supernatants (enriched in ECV, control, or ECV+Flagella, deflagellated) were obtained by pelleting cultures for 30 sec in a Beckman Microfuge B (about 9,000 x g). Pellets were discarded. Time in heading refers to when samples were pipetted into solvent. Aliquots of supernatant from each tube were counted directly without extraction. Extraction and separation of lipids were as previously described. FFAs were scraped from autoradiograms and counted. The percent of label recovered as FFA was found by dividing the FFA count for each sample by the appropriate mean total supernatant (before extraction) counts: 6,916.7 ± 181, control and 10,146.9 ± 531, deflagellated. Total lower phase lipids were determined independently.

A preliminary investigation of the partitioning of the counts during the course of the experiment does not make the fate of the lost counts clear. In the supernatant of control cells (ECVs), about 95% of the total counts were associated with the lower phase Bligh-Dyer lipids, with 3-4% in the upper phase, and less than 1% in the residue. Control cells (the pellet) showed higher percentages of counts associated with the insoluble residue (10%). The possibility that these samples (containing a much larger amount of lipid material and total counts than the supernatant) were less well extracted than the supernatant has not been ruled out. For control cells, the upper Bligh-Dyer phase represented 1-2% of the total, and the lower phase represented 85-88% of the total. Dramatic changes in partitioning of label into the residue or upper phase were not seen during the course of the experiment.

Surface pH Studies of the Flagellar Membrane

According to the Gouy-Chapman-Stern theory, protons are concentrated as counterions to the fixed negative charges of an anionic membrane surface at low ionic strength (McLaughlin et al. 1971). Such a concentration results in a pH which is lower at the surface (up to 3 units) than in the bulk phase. The pH difference between surface and bulk phase (Δ pH) is eliminated at high ionic strength; cations (*e.g.* sodium) replace protons as counterions at the polyanionic surface.

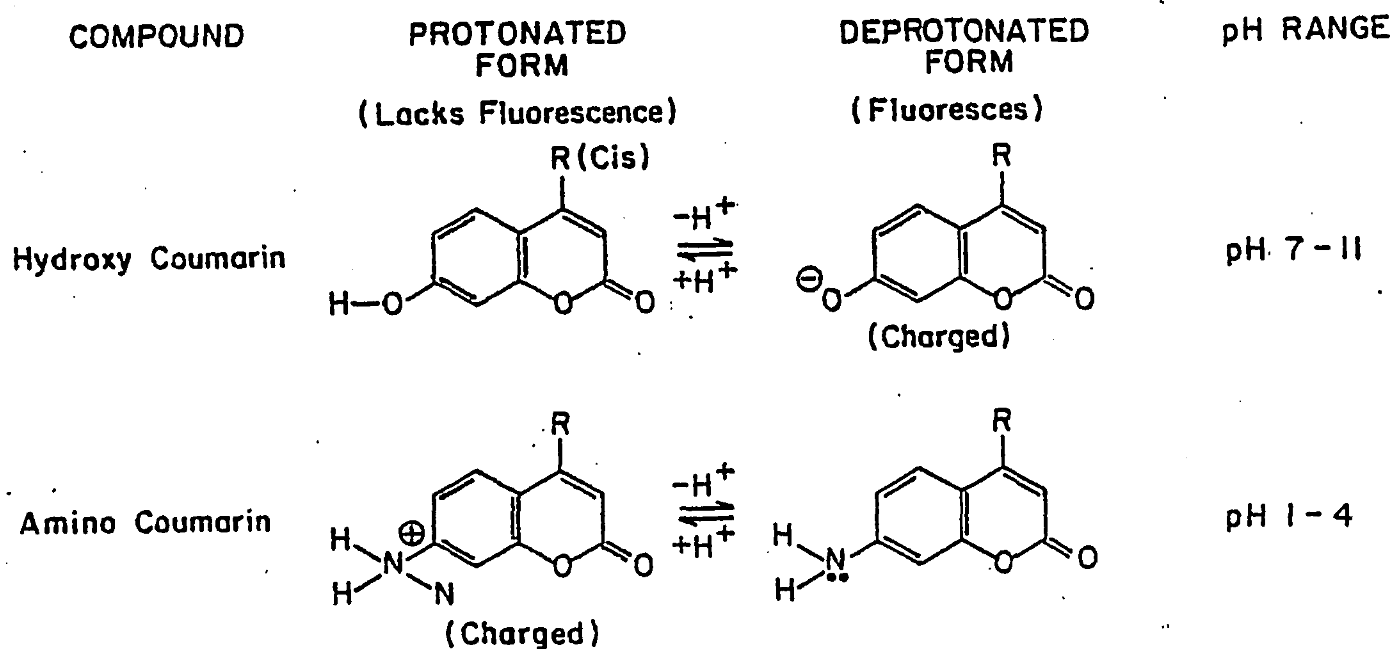
An anionic group with a pK_a in the aqueous range (*e.g.* carboxylate; pK_a=

4.5-4.9) may be protonated at low pH and reduce the surface charge density. This would be particularly true in a membrane made up primarily of fixed anions (*e.g.* sulfates; pK_a = 1.5). The pK_a of a long chain carboxylate exhibits an apparent shift of about 3 units at low ionic strength, so that carboxylates are virtually protonated below pH 7 (bulk phase).

Measurement of the difference between the membrane surface and bulk pH above and below pH 7 provides a means to identify the presence of free fatty acids in the alkyl sulfate membrane. This method has the advantage of avoiding the exposure of the membrane preparation to solvents, which can activate lipolysis.

Alkylated aminocoumarin and hydroxycoumarin indicators (Figure 6) were used to measure the pH at the membrane surface. The apparent pK shifts when the indicators become associated with a membrane or micellar surface (alkylated indicator), compared to when they are in bulk solution (unalkylated indicator). The fluorescence of each indicator at 450 nm is quenched by protonation, and the pK of each indicator in a given surface (be it micelle, liposome, or flagellar membrane) was obtained by titration. Pohl (1976) showed that the indicator reporter remains in the headgroup domain of the bilayer lipids. The percent dissociation of the hydrophobically bound indicator as a function of change in bulk pH is shown for neutral (Triton X-100 micelles) and charged (alkyl sulfate micelles) model membranes in Figure 7. The pK for the hydrophobically bound indicators is shifted from that of the water soluble (unalkylated) forms even in an electrically uncharged system. This shift is equal but in opposite directions because the charged form is bound preferentially to the hydrophobic surface and protonation of one indicator (aminocoumarin) produces a charged

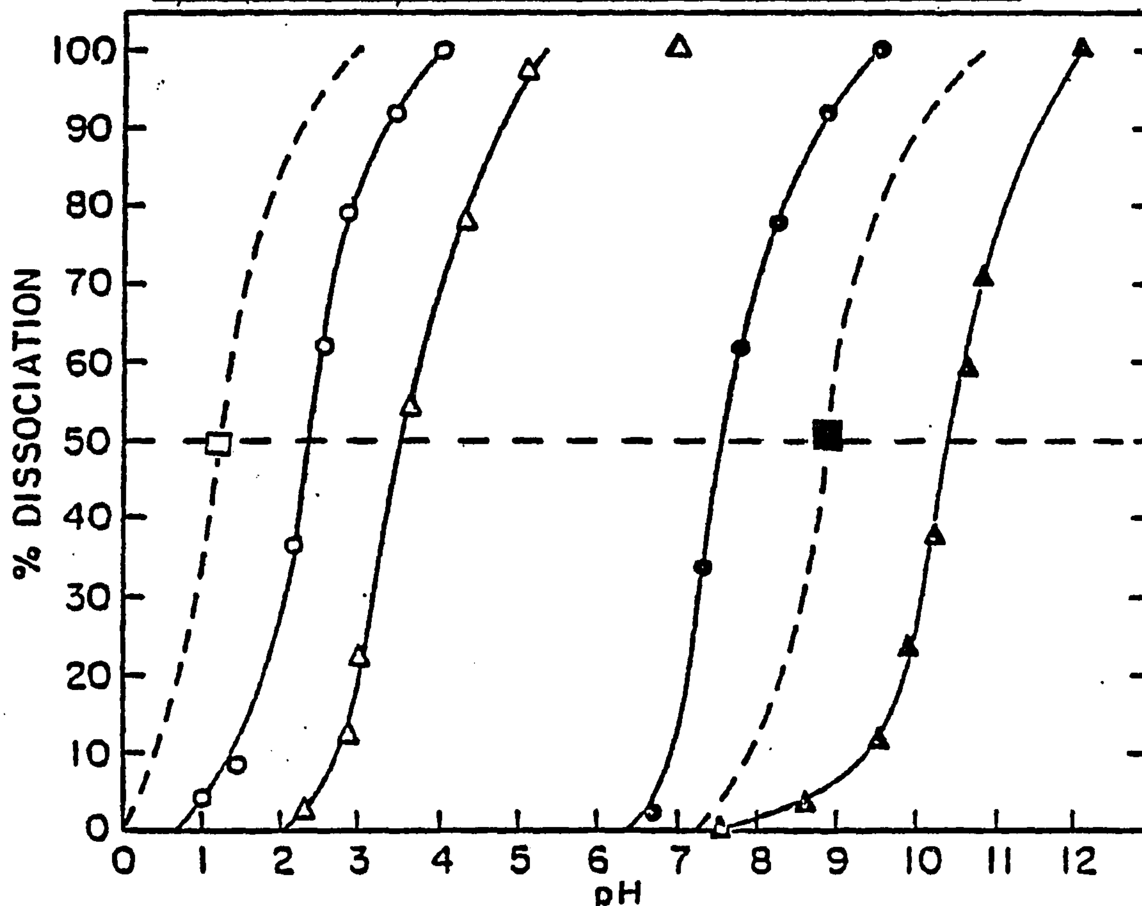
FIGURE 6



Surface pH probes. Alkylated aminocoumarin and hydroxycoumarin dyes are used as indicators to report on the pH at the surface of natural and model membrane systems. Substitution is at position 4 with long chain alkyl groups: R = $-C_{11}H_{23}$ (hydroxycoumarin); R = $-C_{17}H_{35}$ (aminocoumarin). This permits the hydrophobic association of the indicator molecules with micelles and model membranes. The chromophore moiety reports on an area within the Gouy-Chapman Stern layer (within 1 nm from the surface).

FIGURE 7

Dissociation of Coumarin Indicators as a Function of Bulk pH: Free vs. Hydrophobically Bound to Neutral or Anionic Surfaces



The titration behavior of water soluble (non-alkylated) aminocoumarin (o) and hydroxycoumarin (•) indicators is compared to that of alkylated indicators hydrophobically bound to model systems containing surfaces which are neutral (Triton X-100; Fernandez and Fromherz 1977) or negatively charged (sodium oleyl sulfate: cholesterol liposomes; Heller 1982). The pK_a of alkylated aminocoumarin (Δ) and hydroxycoumarin (▲) indicators bound to an anionic surface, or to one which is neutral (□,aminocoumarin; ■ hydroxycoumarin) is shifted with respect to that of the aqueous indicators.

species whereas protonation of the other (hydroxycoumarin) produces an uncharged species (Figure 6). The pH (and charge density) at the surface of various alkyl-suflate based model membranes is compared to that of the flagellar membrane of *O. danica* in Table 8.

Table 8

Surface pH Measurements Using Alkylated Coumarin Indicators

		Alkylated Aminocoumarin			Alkylated Hydroxycoumarin		
		$pK_n = 1.25$ [a]			$pK_n = 8.85$ [a]		
		pK_a	ΔpH	ψ (mV)	pK_a	ΔpH	ψ (mV)
Micelle	Sodium Dodecyl Sulfate (SDS)[a]	3.55	2.3	-134	11.15	2.3	-134
	Sodium Oleyl Sulfate (SOS)	3.60	2.35	-137	10.5	1.65	-95.9
Liposome	SOS:Cholesterol	3.50	2.25	-131	10.5	1.65	-95.9
	Oleate:Cholesterol	--	--	--	10.7	1.8	-106.4
	Oleate:SOS: Cholesterol	1.26	0.01	-0.6	10.3	1.45	-84.3
Natural Membrane of *O. danica*	Flagellar membrane	1.05	-0.20	+11.6	10.0	1.15	-66.8
	Extracellular vesicles	1.0	-0.25	+14.6	9.63	0.78	-45.3

The apparent pK (pK_a) of the alkylated coumarin indicators in a model or natural membrane is shifted by both the membrane's polarity (polarity component), and the charge at its surface (electrostatic component). The polarity component, pK_n has been measured in an uncharged system (Triton X-100)[a]. The electrostatic component, ΔpH, is found from the difference between pK_a and pK_n. The surface potential, (mV) is derived from the equation:

$$\psi = 2.3RT/F \times \Delta pH$$

[a] Fromherz and Fernandez (1977)

DISCUSSION

Free fatty acids are highly disruptive to natural phospholipid membranes in that they discharge proton gradients. Because the chlorosulfolipid membrane is very unlike the familiar phospholipid membrane in a variety of ways, it is not known whether or not they would be disruptive in this system. Relatively high levels of free fatty acids are reported in sulfolipid-containing membranes such as those of *O. danica* (Chen et al 1976), the brown algae (Laur and Liem 1970) or *N. alba* (Anderson et al 1978). Components of these membranes, alkyl or sterol sulfates, are widespread and may be characteristic of acid membranes.

The glycerol backbone of the phosphoglycerides orients the charged headgroup well above the fatty acid chains in phospholipids (Pearson and Pascher 1979). This statement likewise applies to sulfated glycolipids including sphingolipids (Pascher and Sundell 1977a). In contrast, the alkyl sulfates or sterol sulfates have the charged headgroup directly attached to the aliphatic domain. The proximity of these charged groups to the hydrocarbon domain in bilyars is shown by the X-ray structure of cholesterol sulfate (Pascher and Sundell 1977) and sodium dodecyl sulfate (Sundell 1977). Protonated fatty acids thus have what would appear to be a greater capacity to hydrogen bond with these anionic headgroups. Thus the finding that 12 molar percent of the lipids of the flagellar membrane of *O. danica* is free fatty acids is less surprising since it is an alkyl sulfate membrane with a low surface pH.

The two most probable explanations for the occurrence of high levels of free fatty acids (FFAs) in flagellar membrane preparations of *O. danica* are

either that they result from lipolysis during or prior to lipid extraction, or that they are actual components of the natural membrane. It should be noted that an outer membrane or wall is not visible using the electron microscope. It should also be noted that the flagellar membrane is continuous with the plasma membrane and that in another protozoan (*Tetrahymena*) the ciliary and plasma membranes have the same lipid composition (Nozawa and Thompson 1971).

The flagellar membrane of *O. danica* is 70 mol% chlorosulfolipid; phospholipids are entirely absent (Chen et al. 1976). Lipolytic production of free fatty acids from an esterified form of the chlorosulfolipids is unreasonable chemically because (1) there is no free hydroxyl on the sulfolipid structure and (2) a sulfate-carboxylate mixed anhydride is extremely unstable in aqueous solution. Sterols comprise nearly 10% of the total membrane lipids, and FFAs are over 12% (Chen et al. 1976). This leaves a maximum of 8% of other lipids as minor polar and non-polar lipids. One of these is 1(3),2-diacylglyceryl-(3)-O-4'-(N,N,N-trimethyl)homoserine (THAGE) (Brown and Elovson 1974). Lipolytic activity would presumably not result in the *total* loss of the degraded polar lipid, but would leave a residual quantity. Thus the unknown polar lipids of the flagellar membrane would be the most likely sources of FFAs if indeed the latter were generated by lipase activity. There are two steps at which such lipase(s) could be activated: extraction or flagellar detachment.

Does Extraction Activate Lipolysis? Extraction of lipids in higher plants and other cells sometimes activates lipolysis, resulting in production of free fatty acids (Kates and Eberhardt 1957; Kates 1970). A

variety of experimental approaches were therefore taken to determine whether or not this was occuring in *O. danica* preparations, including:

- quantitation and composition of the FFA fraction obtained by extraction after treatment with boiling isopropanol

- extraction after treatment of the flagellar pellet with glutaraldehyde

- testing for the transfer of free fatty acids from flagellar membrane to albumin *prior to extraction*

- demonstration that the surface charge density of the flagellar membrane varies with pH in a manner characteristic of model bilayers containing *free fatty acids*.

Although many phospho- and other lipases are denatured by extracting solvents containing alcohols, several enzymes (such as phospholipase D from sugar beets and runner bean) have proven stable to this treatment (Kates 1972). These enzymes have been inhibited by using either boiling water or alcohol in the extraction procedure (Kates and Eberhardt 1957). Other lipases inactivated by this treatment include a phospholipase A (Notari et al. 1980) and certain lysophospholipases (Thompson 1969). It should be noted that some phospholipase A enzymes are resistant to this treatment (Nishijima et al. 1974;1977).

A preliminary extraction step using boiling isopropanol in the presence of 1 M NaCl prior to extraction with chloroform:methanol failed to reduce the free fatty acid fraction from lipid extracts of *O. danica* flagella. The quantity of free fatty acids per dry weight flagella was essentially the same in treated (0.71%) and in control (0.7%) cells (Table 1). Furthermore, the composition of the fatty acid fraction did not vary in a consistent

manner from that of control flagellar free fatty acids, nor did either resemble the fatty acid composition of two classes of esterified lipids in the cell (Figure 3). Boiling isopropanol treatment also failed to eliminate the FFAs found in lipid extracts of the red alga, *Noctiluca milaris* (Dikarev et al. 1982)

Another approach was to fix pelleted flagella in glutaraldehye prior to extraction. The reagent would not necessarily inactivate a putative lipase, but would be expected to reduce its activity by crosslinking it to the insoluble cell residue, restricting its time in contact with substrate. This should depress the quantities of (putative) lipase-generated free fatty acids. Korn and Weisman (1966) showed that glutaraldehyde fixation of *Acanthamoeba* permitted the recovery of most of the lipids originally labelled with ^{3}H-palmitate in subsequent extraction (and dehydration) steps using ethanol. Recovery was quantitative for the non-polar lipids; 84% of the phospholipids were also extracted.

As shown in table 1, glutaraldehyde crosslinking failed to eliminate or selectively depress the free fatty acid fraction of whole flagella in *O. danica*; however, it was associated with a decrease in the *total* amount of extractable lipid per dry weight flagella. Although FFAs represented about 3% of the weight of flagellar lipids in untreated samples, they made up almost 10% of the extracted lipid weight after glutaraldehyde fixation. This was probably not due to an increase in FFA production, since the overall level of this lipid is low compared to controls. More likely, these results are consistent with the findings of Korn and Weisman (1966) that the polar lipids are less well extracted than nonpolar lipids after glutaraldehyde treatment. Since the chlorosulfolipids of *O. danica* are considerably more

polar than most phospholipids, the differences between polar and nonpolar lipids should be more extreme.

Another way to rule out possible solvent-activated FFA generation would be to determine whether or not these lipids were present *prior* to the extraction step. Albumin transports FFAs in the blood, and has also been used to introduce or remove these compounds *in vitro* (Goto and Mizushima 1978; Johannsson et al. 1981). If free fatty acids are natural membrane components, albumin should be able to bind and remove them from a pelleted membrane phase to the supernatant. This would occur prior to solvent extraction, greatly decreasing the possibility of solvent-activated enzymatic degradation. Although the absence of albumin binding (or association) with free fatty acids would argue against their occurrence in the natural membrane, the converse is not true. For example, albumin "binding" of free fatty acids could result if they were produced artifactually during the deflagellation procedure.

Exposure of whole flagella to albumin was performed in a solution of the culture medium of *O. danica* at pH 4.5; this is well below physiological pH for albumin. These conditions were necessary to stabilize the flagellar membrane (Chen and Haines 1976). Extracellular vesicles, shown by El Maraghy (1982) to have essentially the same lipid composition as the flagellar membrane, become partially solubilized after resuspension and subsequent centrifugation. This can result in a 10 to 20% loss of chloro-sulfolipid and protein (loss of FFAs was not investigated). The effect is most pronounced at alkaline pH, but was significant even at neutral pH (El Maraghy 1982). Thus, maintaining the pH at 4.5 limits the dissolution of the membrane. In control experiments (Table 2), about 5% of the total

free fatty acids were found in the supernatant after repeated cycles of standing, pelleting, and suspension. Sulfolipid loss was not detected in control samples under these conditions. However, the use of albumin at pH 4.5 requires some rather specific considerations regarding its properties at low pH.

The monomeric form of free fatty acids binds to albumin at 2 high affinity sites with high specificity for long chain fatty acids (Goodman 1958) as well as at 4-5 secondary binding sites which are also available to other compounds. Under normal physiological conditions, the molar ratio of free fatty acid to albumin in human plasma ranges from 0.5 to 1.5 (Court, Dunlop, and Leonard 1971) although it can exceed 4.0 after vigorous excercise (Havel, Naimark, and Borchgrevink 1963) or administration of certain drugs. Hydrophobic interactions are believed to account for most of the binding energy with long-chain fatty acids (Ballou et al. 1945; Ray et al. 1966; Spector 1975); however, methyl esters of spin-labelled analogs were bound slightly less tightly than the corresponding carboxylate anion (Morriset, Pownall, and Gotto 1975). This shows that electrostatic or other interactions involving the carboxylate headgroup also play a role.

Albumin transports lysolecithin in the blood; this accounts for over 98% of the lipid-associated phosphorus co-isolated with albumin (Switzer and Eder 1965). Phosphatidylcholine can also be found associated with albumin, although in very small quantities. The association constant for lysolecithin (4.3×10^4, one ligand) is much lower than that for free fatty acids (oleate, 1.1×10^8, first two ligands), including those at secondary sites (4.0×10^6, next 5 ligands) (Klopfenstein1969; 1969a; Goodman 1958). Thus, there is little reason to suppose that albumin would bind substantial amounts of an

esterified precursor to the free fatty acids in preference to free fatty acids.

Although the physiological pH for human albumin occurs within a narrow range around 7.4, the protein has been shown to be stable under considerably more acidic and alkaline conditions. Two types of conformational changes in the albumin molecule (reversible under most conditions) have been documented at low pH: expansion in volume, and the N to F transition.

Changes in certain physical properties of the protein (optical rotation, intrinsic viscosity, flourescence polarization) have been shown to occur at pHs below 4.0 (Foster 1960) and are caused by a molecular expansion in the molecule which is electrostatic in nature (Yang and Foster 1954; Tanford et al. 1955). Expansion is repressed by high ionic strength (Foster 1960) and increases with increasing temperature (Harrington, Johnson, and Ottewill 1956). This effect of temperature is characteristic of undenatured proteins (Harrington, Johnson, and Ottewill 1956). No change in antigenic activity of plasma albumin preparations taken to a pH of 2.0 and then to neutrality was observed (Champagne 1957).

Reversible changes in electrophoretic mobility observed at pH 4.0 and below are now attributed to another phenomenon, the N to F transition, thought to correspond to the protonation of 3 carboxylate groups in the protein (Foster 1960). Changing of the anion in solution to one more tightly bound than chloride (such as thiocyanate), or the presence of SDS (Aoki and Foster 1958) caused a pH shift of the transition to a lower range, whereas tightly bound cations (Aoki 1958) shifted the equilibrium in the opposite direction. This suggests that net charge is important in determining this change in conformation. However, unlike the volume change at low pH, ionic

strength does not seem to influence this behavior. The F, but not N form of albumin has been shown to be a fusogenic agent for small unilamellar vesicles made of phosphatidylcholine (Schenkman et al. 1981; Schenkman et al. 1981a; Garcia, Araujo, and Chaimovitch 1984). This activity was highest at pH 3.5, whereas by pH 4.5 and above, the activity was negligible (Garcia, Araujo, and Chaimovitch 1984). This is further evidence that the conformation of albumin at pH 4.5 is primarily the N form. Both the N to F transition and the volume expansion are reversible, and occur primarily below the pH of this experiment.

Albumin is stabilized against denaturation by free fatty acids and by alkylsulfates such as SDS (Foster 1960); both stabilize the folded, more compact form of albumin (Boyer et al. 1946). The binding of SDS to BSA was found to be independent of solution pH over the range of 4.8 - 6.8 (Reynolds et al. 1970). ^{13}C-NMR studies of oleic acid/BSA complexes showed multiple resonances attributed to the bound carboxylate group of the ligand. No change in chemical shifts were seen as the bulk pH was varied from 6 to 10. However, one resonance shifted as the bulk medium was acidified below pH 6, having an apparent pK_a of ~4 (Parks et al. 1983). There was no evidence for decreased fatty acid binding at low pH in this study. Benson and Hallaway (1970) found the albumin molecule to be most stable against conformational fluctuations in solution (measured by hydrogen-deuterium exchange) in the pH range of 5.0 to 6.5. Addition of SDS at either pH 5 or pH 7 had the effect of decreasing the amount of quickly exchanging hydrogens, increasing the amount of non-exchanging hydrogens, and had little effect on slow exchange. This is again consistent with stabilization of a more compact form for the protein. Hvidt and Wallevik (1972) found similar

results for the effect of detergent binding on albumin, but reported a higher pH for optimal stability of defatted albumin.

Treatment of *O. danica* flagellar membrane with albumin did not result in gross changes in the amount or consistency of the pelleted material, or in morphological changes when observed using transmission electron microscopy. A 3-8 fold increase over control levels of free fatty acids in the supernatant of albumin-treated whole flagella (Table 2) was found. The fatty acids showed a composition similar to that of the corresponding free fatty acids remaining in the pelleted whole flagella, and to control samples (additional unreported data). This suggests that free fatty acids are available for binding to albumin prior to extraction, and hence, that they are not the product of solvent-activated lipolysis.

Another approach for seeking the presence of free fatty acids in the natural flagellar membrane is to compare variations of its surface charge density with pH to that of model membranes containing free fatty acids. Thus, the surface charge density of the membrane is measured above and below the pK of the free fatty acid.

The use of alkylated coumarin pH indicators (shown in Figure 5) to measure surface pH of charged micelles was first introduced by Fromherz (1973). The method was later refined (Fernandez and Fromherz 1977) to discriminate between two components to the observed shifts in the indicator's appparent pK_a upon association with a charged surface:

1. the preferential binding of the uncharged indicator form to a low dielectric surface (polarity component)

2. the pH difference between the charged surface and the bulk medium (electrostatic component).

The difference between the pH at the surface and bulk pH can be used to calculate the surface charge density of a membrane (see legend to Table 8).

Hydroxycoumarin (pK_a=7.75) is uncharged in its protonated form, whereas aminocoumarin (pK_a=2.35) is uncharged when de-protonated. The binding effect for the pair after association with an uncharged surface (Triton X-100 micelles) yields pK_a shifts in equal and opposite directions for these two indicators (the apparent pK_a becomes 8.85 and 1.25, respectively). The electrostatic component shifts the apparent pK_a equally and in the same direction for both indicators (Fernandez and Fromherz 1977). Association of the indicators with a polyanionic surface such as SDS micelles causes composite shifts, representing the sum of the binding and electrostatic components.

Although the sulfate headgroup is expected to remain dissociated over most extreme ranges of pH, the carboxylate group has a pK_a of about 4.7. The apparent pK for long chain fatty acids can be much higher; in myristyl sulfate micelles, FFAs had an apparent pK_a of 7.75 (Ptak et al. 1980). For model membranes consisting of SOS (sodium oleylsulfate) or SOS and cholesterol, at both high and low ranges of bulk pH, the surface pH was about 2.3 units lower (Table 8) than that of the bulk phase. Free fatty acids (model membranes of SOS/cholesterol/FFA 1:1:2) contributed to the surface potential at high (7-12) but not at low (0-7) pH. The behavior of the

surface potential of the flagellar membrane (near neutral at low pH but negative at high pH) was similar to that of fatty acid-containing model membranes (Table 8). This is consistant with the presence of free fatty acids in the flagellar membrane preparation.

The surface pH measurements, in combination with the data from lipase inhibition during solvent extraction, glutaraldehyde fixation prior to extraction, and albumin treatment of the membrane, suggest that FFAs are present in the isolated flagellar membrane of *O. danica*. But are free fatty acids actually present in the *natural* membrane? The effect of flagellar detachment was investigated to determine whether the isolation procedure itself was contributing to the FFA levels.

Does Flagellar Detachment Activate Lipolysis? High levels of lipolytic activity would be required if this activity were the sole explanation for the flagellar membrane lipid composition (12 mol% FFAs with 5 unknown polar lipids representing less than 8 mol% "residual" polar lipids). Widespread lipolysis can occur on a rapid time scale in some systems, *i.e.,* wounding of certain plant tubers and storage organs (Galliard 1978). Could the flagellar detachment procedure be analogous to wounding in these plant systems?

Many types of algae shed their flagella in response to physical or chemical stress, including unfavorable conditions of temperature, pH, the presence of alcohols, detergents, or toxic materials (Lewin et al. 1982). This may protect the organism against predators by rapidly freeing flagella which had become irreversiblly attached to a surface. The flagella can represent a large percentage of the cell surface area (10% in

Chlamydomonas moewusii; a greater percentage in *O. danica*). Its loss could reduce permeable surface area under adverse conditions (Lewin, Lee, and Fang 1982). Lead has been shown to be less toxic to mechanically deflagellated cells than to normal flagellate cells of *Platymonas* (Hessler 1974), suggesting a selective advantage.

Flagella are obtained from *O. danica* using a simple mechanical procedure; detachment is followed by flagellar regeneration within 6 hours (Rosenbaum and Child 1967). Therefore, flagellar detachment does not cause long-term damage to the cultures. In order for regeneration to take place, new membrane is synthesized. Free fatty acids, if formed, would remain in the discarded membrane. Since the organism releases extracellular vesicles (ECVs) and does not incorporate membrane or membrane components during deflagellation, lipase activity has no obvious utility and would seem to be disadvantageous. In contrast, the widespread lipolysis caused by wounding of the potato causes the rapid destruction of a large part of the tuber. The lipolytic activity presumably provides nutrition for the germinating seedling under wounding circumstances.

The experimental results reported herein support the argument against lipase activation by flagellar detachment. No significant increase in FFA levels with time after deflagellation was seen in either the supernatant (containing flagella + extracellular vesicles, or ECVs: Table 7) or in unpelleted material (containing cell bodies + flagella + ECVs: Table 6). Differences in the quantity of label found as free fatty acid in cells after flagellar detachment, and in control (untreated) cells were also not observed (Table 6). Acid hydrolysis of the total lipids of whole cells led to a significant increase (from 8% to 65% of the total in one case) in the total

lipid counts isolated as free fatty acids. FFA levels would be expected to rise after flagellar detachment if such treatment provoke widespread lipolysis. The absence of such a rise suggests that the FFAs are not derived from lipolysis during detachment.

One assumption made here is that the cold treatment (1 to 3 hours at 0 °C, preceeded by centrifugation during harvest at 4 °C) does not activate lipolysis and thereby free fatty acid production. Both control and experimental samples were subjected to the same cold treatment in the deflagellation experiments. Prolonged exposure to cold causes changes in membrane lipids (White and Somero 1982). In some specialized cells (brown fat cells), free fatty acids may play a role in thermogenesis by uncoupling oxidative phosphorylation (Nicholls 1979).

In *Tetrahymena pyriformis*, a distinct pattern of lipid changes in microsomal membranes have been shown during various stages of cold acclimation (Martin and Thompson 1978). The cilia undergo an early set of compositional changes independent of such changes in the microsomal membranes (*ie*, not due to ciliary importation of fatty acids) during chilling. In the first 4 hours of chilling, the overall ciliary fatty acid compositon remained the same as for untreated cells, but they were redistributed among the different phospholipid classes in the membrane (Ramesha and Thompson 1984). This redistribution of fatty acids could also be demonstrated in isolated cilia *in vitro*, in which case it requires the presence of ATP and CoA. The authors commented upon the absence of free fatty acids and lysophosphatides under these conditions (4 hours after chilling) even though they had been specifically sought (Ramesha and Thompson 1984). The timescale for these changes in *Tetrahymena* is

similar to the length of time for which *O. danica* was chilled prior to deflagellation. The absence of FFAs in *Tetrahymena* during this period suggests that cold treatment *per se* does not normally lead to the liberation and persistance of large quantities of free fatty acids, as are found in *O. danica*

Even more significant is the fact that free fatty acids are found in the membranous extracellular vesicles extruded by *O. danica* The overall lipid composition of the ECVs is similar to that of the flagellar membrane. These vesicles arise from the entire plasma membrane of *O. danica* including the flagellar membrane. The ECVs are not subjected to prolonged cold treatment, so it seems reasonable to infer that flagellar free fatty acids are also not produced by exposure to cold.

One could perhaps argue that the FFAs isolated with the flagellar membrane originated from the extruded ECVs that are subjected to lipolysis in the medium. The observation that the overall lipid composition, and the sulfolipid, sterol, and fatty acid composition of the ECVs are each found to approximate those of the flagellar membrane (El Maraghy 1982) makes this unlikely.

In conclusion, it has been shown that the free fatty acids of the flagellar membrane of *O. danica* are not the result of solvent-activated lipase, nor do they result from flagellar detachment. It would seem that they are therefore present in the natural chlorosulfolipid membrane.

How can the FFAs found in the cell-surface membranes of *O. danica* form a viable membrane in this sulfolipid system, whereas they are disruptive to natural phospholipid membranes?

The free fatty acids constitute 12 mole% of the membrane, and 12% of

the surface area as well, since the the flagellar membrane lipids (alkyl sulfates) are mono-chains. Were the fatty acids to patch or phase separate, micelle or oil droplet formation would be likely. It is therefore reasonable to conclude that they are interspersed among the chlorosulfolipids. Hargreaves and Deamer (1978) noted a requirement for "spacer" molecules (sterols, alkanols) when forming liposomes from alkyl sulfate or other highly anionic lipids. Protonated fatty acids could act as "spacer" molecules in this system. Hauser et al. (1979) have showed that long chain free fatty acids tend to cluster in phosphatidylcholine vesicles near neutral pH, and are not randomly distributed until >pH 11. No more than 10 mole% fatty acids were incorporated in these studies.

Gebicki and Hicks (1973) first showed that stable preparation of liposomes consisting solely of unsaturated FFAs could be formed between pH 7 to 9.6. Hargreaves and Deamer (1978) showed that bilayers could be formed regardless of chain length and saturation, provided that the FFAs were above their transition temperature. Above pH 10, the FFAs were micellar; below pH 7, they formed oil droplets. Studies using ^{13}C-NMR to monitor the the carboxylate carbon and the stability of FFA liposomes as a function of bulk pH showed distinct protonation states associated with fatty acids in the three phases (Heller 1982): oil droplets (fully protonated), micelles (fully deprotonated), liposomes (near pK of carboxylate; 20 - 80% dissociation). Thus the titration of 50 mM fatty acids from pH 5 to 10 displays two inflection points or two "apparent pKs". This implies that three molecular species are present although there is only one carboxylate.

An acid-anion is a dimer in which one proton is shared by two carboxylate groups. Distribution of the resulting single anionic charge over

the four carboxylate oxygens stabilizes the structure. Smith and Tanford (1973) stated "hydrogen bonds between R-COO$^-$ and R-COOH are extraordinarily stable when carboxyl groups are attached to long alkyl chains". The pK for the shared proton is affected by forces that juxtapose the acid and anion, such as the hydrophobic interactions found in membranes. Molecular geometry can also stablize the acid-anion, as was elegantly shown for maleic acid and *rac*-2,3-di-*tert*-butyl succinate. These dicarboxylates have unusually low first pKs and/or high second pK values, *ie,* an extended pH range during which one proton is present (Westheimer and Benfey 1956). Acid-anion formation in the headgroup region has been proposed as a universal feature stabilizing acid membranes (Haines 1983).

Hargreaves and Deamer's (1978) findings that liposomes could be formed from sodium dodecyl sulfate (SDS) provided that "spacer" molecules were present was attributed to a requirement for separation of repulsive charges. If FFA serve this role, they could conceivably further stabilize the membrane through sulfate-carboxylate mixed acid-anion formation. At any rate, at pH 4.5 (optimal for growth of *O. danica*), the FFAs would be fully protonated. These, with the sterols, would function as "spacers".

The structure of the phospholipids imposes a unique geometry; the phosphate headgroup is held at a higher level in the membrane than the headgroups of alkyl sulfates, sterol sulfates, and unesterified long chain carboxylates. The unique orientation of sulfolipids may permit interactions, such as mixed acid-anions, that are not possible in phospholipid membranes. The necessity for FFAs to act as "spacers" may make them less "available" or "free" in the sulfolipid membrane, compared to one which is phospholipid-based. Presumably, lower flip-flop rates for free fatty acids

would occur in the sulfolipid membrane. If the carboxylates of the free fatty acids strongly interact with the sulfate esters of sulfolipids through acid-anion formation, their tendency towards dissipation of proton gradients (through binding or releasing of additional protons via the carboxylate headgroup) could become thermodynamically unfavored.

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